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(54) Title: METHOD FOR MOLECULAR STAGING OF PROSTATE CANCER

(57) Abstract

    A method for enhancing the detection of prostate specific antigen in a biological sample comprising (a) extracting mRNA from the sample; (b) contacting the mRNA from step (a) with reverse transcriptase under conditions allowing for the production of cDNA; (c) contacting the cDNA from step (b) with a pair of reverse transcriptase polymerase chain reaction primers capable of specifically hybridizing with DNA encoding prostate specific antigen wherein one such primer is an oligonucleotide of 12 to 30 nucleotides in length and comprises the sequence 5-CACCCATCCTA-3' and wherein the second such primer is an oligonucleotide of 12 to 30 nucleotides in length and comprises the sequence 5'-TCCAGCCACGAC-3'; and wherein at least one of the primers is covalently linked to a modified digoxigenin molecule and under conditions allowing for extension of the primers; and (d) detecting the resulting amplified DNA.

METHOD FOR MOLECULAR STAGING OF PROSTATE CANCER

5   This application is a continuation in part of U.S. Serial No. 08/229,391, filed April 15, 1994, the contents of which are hereby incorporated by reference. This invention was made with support under Grant No. CA58089 from the National Cancer Institute, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in the
10 — invention.

Background of the Invention

15   Throughout this application, various publications are referenced. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to
20 which this invention pertains.

   Approximately 60% of newly diagnosed patients with prostate cancer will have established metastasis at the time of diagnosis (2). Once the disease has spread to
25 distant rites, the overall prognosis is poor (3,4). Organ-confined prostate cancer should be curative upon removal of the gland, however screening modalities to assess early metastases often fail to identify a significant subset of patients with locally invasive
30 disease (involving penetration of the capsule or seminal vesicle). Recent studies report that up to 40-50% of patients who were thought to have clinically localized disease were found to be understaged subsequent to radical surgery (5,6,7). This failure
35 rate creates a clinical dilemma since operative therapy is not the appropriate treatment modality for these patients. Clearly, development of a more sensitive

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means to identify patients with micrometastic, locally invasive disease is warranted.

Prostate cancer metastases are more frequently found in the pelvic lymph nodes and on bone and these are sites surveyed most aggressively in patients diagnosed with prostate cancer. The involvement of draining lymph nodes in the dissemination of this cancer is expected, given our understanding of solid tumor behavior. The means by which the prostate cancer cells target the bone is debated, but suspected to involve blood vessels to the lower spine, specifically the vertebral venous plexus (8,9). This spread presupposes that prostate cancer cells are shed into the blood stream and implies a role for hematogenous dissemination in prostate cancer progression.

Indeed, a preliminary study showing that prostate cells can be putatively identified in blood specimens of patients with metastatic prostate cancer supports the concept of blood-borne metastasis (10).

This previous study reported that cells synthesizing prostate specific antigen (PSA) were present in the circulating blood of patients with prostate specific antigen (PSA) were present in the circulating blood of patients with prostate cancer metastases. Since the expression of this protein is restricted to epithelial cells of the prostate gland, the detection of PSA synthesizing cells in the circulation indicates an unexpected and potentially abnormal situation. Here, we describe our development of an extremely sensitive "enhanced" PCR-based assay that allows us to identify PSA-synthesizing cells even when they are highly diluted in a population of peripheral lymphocytes. When this assay was applied to RNA extracted from peripheral blood cells of prostate cancer patients, it

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enabled us to distinguish the overwhelming majority of patients with overt metastatic disease as well as patients with locally invasive tumors that were understaged by conventional screening modalities (i.e. digital rectal exam, CT-scan and/or endorectal MRI). The remarkable accuracy of this assay in upstaging patients with apparent clinically localized disease could eliminate a significant number of prostate cancer patients from unnecessary operations and potentially increase the cure rate in patients who are treated by radical prostatectomy. Since this simple assay involves the molecular detection of a prostate-specific gene product, it represents the first reported instance of molecular staging of a solid human tumor.

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Summary of the Invention

The subject method provides a method for enhancing the detection of prostate specific antigen in a biological sample suspected of containing prostate specific antigen which comprises:

(a) extracting mRNA from the sample;

(b) contacting the mRNA from step (a) with reverse transcriptase under conditions allowing for the production of cDNA;

(c) contacting the cDNA from step (b) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; and wherein at least one of the primers is covalently linked to a suitably modified digoxigenin molecule, under conditions allowing for

hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(d) contacting the double stranded duplex from step (c) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen and comprising a suitably modified digoxigenin molecule; and

(e) detecting the suitably modified digoxigenin molecule from the cDNA from step (d) thereby detecting prostate specific antigen.

The subject invention also provides a method for detecting prostate specific antigen in a biological sample suspected of containing prostate specific antigen which comprises:

(a) extracting mRNA from the sample;

(b) contacting the mRNA from step (a) with reverse transcriptase under conditions allowing for the production of cDNA;

(c) contacting the cDNA from step (b) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the

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nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3'
wherein each of X, X', Y, and Y' may be
present or absent, but if present X is A,
GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,
AACACAGA, TAACACAGA, ATAACACAGA,
AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA;
X' is C, AC, GAC, TGAC, ATGAC, GATGAC,
TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or
CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT,
or TCTGTG; and Y' is C, CT, CTC, CTCA,
CTCAT, CTCATG, or CTCATGC; under conditions
allowing for hybridization of both primers
to any cDNA encoding prostate specific
antigen so as to obtain a double stranded
duplex comprising the cDNA hybridized to the
primers;

(d)   contacting the double stranded duplex from
step (c) with DNA polymerase under
conditions allowing for the extension of the
primers to produce cDNA encoding prostate
specific antigen; and

(e)   detecting the cDNA from step (d) thereby
detecting prostate specific antigen.

The subject invention also provides a method of
diagnosing prostate cancer in a subject which
comprises:

(a)   obtaining a biological sample from the
subject;

(b)   extracting mRNA from the sample;

(c)   contacting the mRNA from (b) with reverse
transcriptase under conditions allowing for

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the production of cDNA;

(d)   contacting the cDNA from step (c) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(e)   contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f)   detecting the cDNA from step (e) thereby diagnosing prostate cancer in the subject.

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The subject invention further provides a method of determining the stage of prostate cancer in a subject which comprises:

5   (a)   obtaining   a   biological   sample   from   the
                 subject;

           (b)   extracting mRNA from the sample;

10 --   (c)   contacting the mRNA from (b) with reverse
                 transcriptase under conditions allowing for
                 the production of cDNA;

           (d)   contacting the cDNA from step (c) with a
15   pair of reverse transcriptase polymerase
                 chain   reaction   oligonucleotide   primers
                 capable of specifically hybridizing with DNA
                 encoding prostate specific antigen, wherein
                 one such primer is an oligonucleotide 12 to
20   30   nucleotides   in   length   and   having   the
                 sequence   5'-X-CACCCCATCCTA-Y-3'   and   the
                 second such primer is an oligonucleotide 12
                 to 30 nucleotides in length and having the
                 nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3'
25   wherein each of X, X', Y, and Y' may be
                 present or absent, but if present X is A,
                 GA,   AGA,   CAGA,   ACAGA,   CACAGA,   ACACAGA,
                 AACACAGA,   TAACACAGA,   ATAACACAGA,
                 AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA;
30   X'   is   C,   AC,   GAC,   TGAC,   ATGAC,   GATGAC,
                 TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or
                 CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT,
                 or TCTGTG; and Y' is C, CT, CTC, CTCA,
                 CTCAT, CTCATG, or CTCATGC; under conditions
35   allowing for hybridization of both primers
                 to   any   cDNA   encoding   prostate   specific
                 antigen so as to obtain a double stranded

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duplex comprising the cDNA hybridized to the primers;

(e) contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f) detecting the cDNA from step (e) thereby determining the stage of prostate cancer in the subject.

The subject invention further provides an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' wherein each of X and Y may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; and Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG.

The subject invention also provides an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X' and Y' may be present or absent, but if present X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC.

Finally, the subject invention provides a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers for detecting the expression of the prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30

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nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present
5   X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT,
10   ─ CTCATG, or CTCATGC.

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Brief Description of the Figures

Figure 1. RT-PCR assay identifies expression of PSA in the LNCaP cell line in a specimen of human prostate cancer. Total RNA was extracted from LNCaP cells and from a human prostate tumor. Aliquots were assayed for the presence of the diagnostic 710 bp PSA-specific PCR DNA fragment following treatment with oligo-dT with (+RT) or without (-RT) reverse transcriptase. The 710 bp PSA fragment was detected by UV transillumination of the ethidium bromide stained agarose gel following electrophoresis.

Figure 2. Sensitivity of RT-PCR assay determined by analysis of diluted LNCaP cells. RNA was extracted from serial dilutions of LNCaP cells in 10^6 cultured B lymphocytes and was assayed by RT-PCR for PSA. The 710 bp PSA-PCR fragment could be amplified only from specimens containing LNCaP cells (top panel). Human G3PDH primers were utilized in a parallel reaction and these primers amplified the appropriate 983 bp fragment from all specimens (lower panel).

Figure 3. Inability to detect PSA synthesizing cells in peripheral blood lymphocytes of noncancer male patients. Total RNA was extracted from the lymphocyte fraction of 8 young males with no history of urological disease and age-matched BPH patients. These RNAs were assayed by RT-PCR with PSA primers. None of these specimens yielded a positive fragment by ethidium bromide visualization (top

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panel) or by the enhanced visualization
method (not shown). All specimens did yield
an appropriate reaction product when G3PDH
primers were utilized in the PCR reaction.

5

Figure 4. Inability to detect PSA synthesizing cells
in peripheral blood lymphocytes of females.
Total RNA was extracted from the lymphocyte
fraction of 6 females. These RNAs were

10   — assayed by RT-PCR using PSA primers. None
of these specimens yielded a positive
fragment by ethidium bromide visualization
(top panel) or by the enhanced method (now
shown). All specimens did yield an

15   appropriate reaction product when G3PDH
primers were utilized in the PCR reaction
(bottom panel).

Figure 5. Comparison of RT-PCR and enhanced RT-PCR for
20   detection of PSA-synthesizing cells in the
peripheral blood of 23 individual prostate
cancer patients. Top panels; total RNA was
extracted from peripheral blood lymphocytes
of patients with localized and metastatic

25   prostate cancer. This RNA was assayed by
RT-PCR for PSA. Three specimens, as well as
the LNCaP and prostate tumor control RNA
demonstrate the presence of this fragment
following ethidium bromide staining of the

30   agarose gel. Bottom panels; demonstrate
enhanced detection of the 710 bp PSA
fragment. Luminescent substrate and
autoradiography allowed the detection of 7
additional positive specimens in this group

35   of patients.

Figure 6. Nucleic acid sequence of the cDNA encoding
the PSA protein.

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Detailed Description of the Invention

This invention provides a method of determining the
stage of prostate cancer in a human subject which
comprises: collecting a sample of peripheral blood
5   from a human subject; extracting mRNA molecules from
the peripheral blood; amplifying a cDNA molecule
encoding the PSA protein which comprises: (i)
contacting the mRNA molecule extract with a nucleic
acid molecule primer capable of specifically
10   hybridizing to a nucleic acid sequence site included
within the mRNA encoding the PSA protein under
conditions allowing the primer to specifically
hybridize to the mRNA encoding the PSA protein; (ii)
contacting the composition of (i) with reverse
15   transcriptase under conditions allowing the reverse
transcriptase to transcribe a single stranded cDNA
molecule copy of the mRNA molecule encoding the PSA
protein; (iii) heat denaturing the cDNA and mRNA
molecule of composition (ii); (iv) contacting the
20   composition of (iii) with a first primer capable of
specifically hybridizing to a first unique nucleic
acid molecule sequence site located on the cDNA
molecule complementary to the mRNA encoding the PSA
protein under conditions allowing the first primer to
25   specifically hybridize with the first unique nucleic
acid molecule sequence site; (v) contacting the
composition of (iv) with DNA polymerase under
conditions allowing the DNA polymerase to synthesize
a double stranded cDNA molecule encoding the PSA
30   protein using the first primer to initiate synthesis
and the single-stranded cDNA molecule as a template;
(vi) heat denaturing the double-stranded cDNA molecule
of composition (v); (vii) contacting the composition
of (vi) with the first primer and a second nucleic
35   acid molecule primer capable of specifically
hybridizing with a second unique nucleic acid molecule

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sequence site wherein the second unique nucleic acid
sequence site is located on the other cDNA strand 3'
of the first unique site under conditions allowing the
first and second primers to specifically hybridize
with the first and second unique sequences,
respectively, of the cDNA molecule encoding the PSA
protein; (viii) contacting the composition of (vii)
with DNA polymerase under conditions allowing the DNA
polymerase to synthesis cDNA molecules encoding the
PSA protein using the first or second primers to
initiate synthesis and the heat denatured cDNA
molecule encoding the PSA protein as a template; and
(ix) repeating steps (vi) through (viii) for at least
twenty five times; and detecting the presence of the
cDNA molecule encoding the PSA protein and thereby
determining the stage of prostate cancer.

As used herein, the phrase "specifically hybridizing"
means the ability of a nucleic acid molecule to
recognize a nucleic acid sequence sufficiently similar
to its own so as to form double-helical segments
through hydrogen bonding between complementary base
pairs. As used herein, a "unique sequence" is a
sequence specific to only the nucleic acid molecules
encoding the PSA protein.

The collection of blood from a patient is described in
the Experimental Details. An adequate blood sample is
5-8 cc of peripheral blood. Although venous
collection is discussed, peripheral blood can be drawn
from arteries using methods known to one of skill in
the art.

An example of one mRNA extraction procedure is
provided in the Experimental Details, but the
extraction of mRNA from a blood sample is well known
to one of skill in the art and other methods can be

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used.

The preferred DNA polymerase is Taq Polymerase which
is commercially available and has the additional
5   benefit of retaining activity after exposure to DNA
denaturing temperatures.

The determination that a human subject has PSA
expressing cells circulating in the subjects
10 -- peripheral blood can be made by detecting a cDNA
product using the Method described hereinabove that
consists of a base pair size predicted by the primers
used and the known sequence of cDNA encoding the PSA
protein. If primers other than those described in the
15   Experimental Details are used, verification of the
cDNA product produced should be done by sequencing the
cDNA product.

This invention provides a method of determining the
20   stage of prostate cancer in a human subject which
comprises: collecting a sample of peripheral blood
from a human subject; extracting mRNA molecules from
the peripheral blood; amplifying a cDNA molecule
encoding the PSA protein which comprises: (i)
25   contacting the mRNA molecule extract with a nucleic
acid molecule primer capable of specifically
hybridizing to a nucleic acid sequence site included
within the mRNA encoding the PSA protein under
conditions allowing the primer to specifically
30   hybridize to the mRNA encoding the PSA protein; (ii)
contacting the composition of (i) with reverse
transcriptase under conditions allowing the reverse
transcriptase to transcribe a single stranded cDNA
molecule copy of the mRNA molecule encoding the PSA
35   protein; (iii) heat denaturing the cDNA and mRNA
molecule of composition (ii); (iv) contacting the
composition of (iii) with a first primer capable of

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specifically hybridizing to a first unique nucleic
acid molecule sequence site located on the cDNA
molecule complementary to the mRNA encoding the PSA
protein under conditions allowing the first primer to
specifically hybridize with the first unique nucleic
acid molecule sequence site; (v) contacting the
composition of (iv) with DNA polymerase under
conditions allowing the DNA polymerase to synthesize
a double stranded cDNA molecule encoding the PSA
protein using the first primer to initiate synthesis
and the single-stranded cDNA molecule as a template;
(vi) heat denaturing the double-stranded cDNA molecule
of composition (v); (vii) contacting the composition
of (vi) with the first primer and a second nucleic
acid molecule primer capable of specifically
hybridizing with a second unique nucleic acid molecule
sequence site wherein the second unique nucleic acid
sequence site is located on the other cDNA strand 3'
of the first unique site under conditions allowing the
first and second primers to specifically hybridize
with the first and second unique sequences,
respectively, of the cDNA molecule encoding the PSA
protein; (viii) contacting the composition of (vii)
with DNA polymerase under conditions allowing the DNA
polymerase to synthesis cDNA molecules encoding the
PSA protein using the first or second primers to
initiate synthesis and the heat denatured cDNA
molecule encoding the PSA protein as a template; and
(ix) repeating steps (vi) through (viii) for at least
twenty five times; and detecting the presence of the
cDNA molecule encoding the PSA protein which further
comprises: loading the composition of step (ix) onto
a gel; electrophoresing the cDNA into the gel with a
DNA ladder; ethidium bromide staining the DNA inside
the gel; visualizing the ethidium bromide stained DNA
under ultraviolet light; and detecting the presence of
the cDNA molecule encoding the PSA protein and thereby

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determining the stage of prostate cancer.

This invention provides a method of determining the
stage of prostate cancer in a human subject which
5   comprises: collecting a sample of peripheral blood
from a human subject; extracting mRNA molecules from
the peripheral blood; amplifying a cDNA molecule
encoding the PSA protein which comprises: (i)
contacting the mRNA molecule extract with a nucleic
10 ⎯ acid molecule primer capable of specifically
hybridizing to a nucleic acid sequence site included
within the mRNA encoding the PSA protein under
conditions allowing the primer to specifically
hybridize to the mRNA encoding the PSA protein; (ii)
15   contacting the composition of (i) with reverse
transcriptase under conditions allowing the reverse
transcriptase to transcribe a single stranded cDNA
molecule copy of the mRNA molecule encoding the PSA
protein; (iii) heat denaturing the cDNA and mRNA
20   molecule of composition (ii); (iv) contacting the
composition of (iii) with a first primer capable of
specifically hybridizing to a first unique nucleic
acid molecule sequence site located on the cDNA
molecule complementary to the mRNA encoding the PSA
25   protein under conditions allowing the first primer to
specifically hybridize with the first unique nucleic
acid molecule sequence site; (v) contacting the
composition of (iv) with DNA polymerase under
conditions allowing the DNA polymerase to synthesize
30   a double stranded cDNA molecule encoding the PSA
protein using the first primer to initiate synthesis
and the single-stranded cDNA molecule as a template;
(vi) heat denaturing the double-stranded cDNA molecule
of composition (v); (vii) contacting the composition
35   of (vi) with the first primer and a second nucleic
acid molecule primer capable of specifically
hybridizing with a second unique nucleic acid molecule

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sequence site wherein the second unique nucleic acid sequence site is located on the other cDNA strand 3' of the first unique site under conditions allowing the first and second primers to specifically hybridize with the first and second unique sequences, respectively, of the cDNA molecule encoding the PSA protein; (viii) contacting the composition of (vii) with DNA polymerase under conditions allowing the DNA polymerase to synthesis cDNA molecules encoding the PSA protein using the first or second primers to initiate synthesis and the heat denatured cDNA molecule encoding the PSA protein as a template; and (ix) repeating steps (vi) through (viii) for at least twenty five times; and detecting the presence of the cDNA molecule encoding the PSA protein which comprises: adding digoxigenin-labeled nucleotides in step (iv); loading the composition of step (ix) which contains digoxigenin-labeled nucleotides onto a gel; electrophoresing the cDNA into the gel with a digoxigenin-labeled DNA ladder; transferring and fixing the DNA in the gel onto a membrane; contacting the DNA on the gel with an antibody which specifically binds digoxigenin, wherein the antibody is linked to an enzyme capable of directly or indirectly activating a chemiluminescent substrate; contacting the antibody bound DNA with the activatable chemiluminescent substrate; visualizing the presence of labeled DNA by exposing the membrane to a light sensitive device; and detecting the presence of the cDNA molecule encoding the PSA protein and thereby determining the stage of prostate cancer.

A preferred embodiment of the method to determining the stage of prostate cancer in a human subject comprises an enzyme capable of activating a chemiluminescent substrate which may be alkaline phosphatase, a the chemiluminescent substrate which

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may be 4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxetane-3,2-adamantane) disodium salt, and a light sensitive device which may be X-ray film. Other light sensitive devices are known to those of skill in the art for recording labeled nucleotides, whether they be digoxigenin or radioactively labeled.

This invention provides a method of determining the stage of prostate cancer in a human subject which comprises: collecting a sample of peripheral blood from a human subject; extracting mRNA molecules from the peripheral blood; amplifying a cDNA molecule encoding the PSA protein which comprises: (i) contacting the mRNA molecule extract with a nucleic acid molecule primer capable of specifically hybridizing to a nucleic acid sequence site included within the mRNA encoding the PSA protein under conditions allowing the primer to specifically hybridize to the mRNA encoding the PSA protein; (ii) contacting the composition of (i) with reverse transcriptase under conditions allowing the reverse transcriptase to transcribe a single stranded cDNA molecule copy of the mRNA molecule encoding the PSA protein; (iii) heat denaturing the cDNA and mRNA molecule of composition (ii); (iv) contacting the composition of (iii) with a first primer capable of specifically hybridizing to a first unique nucleic acid molecule sequence site located on the cDNA molecule complementary to the mRNA encoding the PSA protein under conditions allowing the first primer to specifically hybridize with the first unique nucleic acid molecule sequence site, wherein the first primer comprises a nucleic acid molecule sequence of at least 15 nucleic acids that is the same or substantially the same as the nucleic acid sequence shown in Figure 6; (v) contacting the composition of (iv) with DNA polymerase under conditions allowing the DNA

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polymerase to synthesize a double stranded cDNA molecule encoding the PSA protein using the first primer to initiate synthesis and the single-stranded cDNA molecule as a template; (vi) heat denaturing the double-stranded cDNA molecule of composition (v); (vii) contacting the composition of (vi) with the first primer and a second nucleic acid molecule primer capable of specifically hybridizing with a second unique nucleic acid molecule sequence site wherein the second unique nucleic acid sequence site is located on the other cDNA strand 3' of the first unique site under conditions allowing the first and second primers to specifically hybridize with the first and second unique sequences, respectively, of the cDNA molecule encoding the PSA protein; (viii) contacting the composition of (vii) with DNA polymerase under conditions allowing the DNA polymerase to synthesis cDNA molecules encoding the PSA protein using the first or second primers to initiate synthesis and the heat denatured cDNA molecule encoding the PSA protein as a template; and (ix) repeating steps (vi) through (viii) for at least twenty five times; and detecting the presence of the cDNA molecule encoding the PSA protein and thereby determining the stage of prostate cancer.

This invention provides a method of determining the stage of prostate cancer in a human subject which comprises: collecting a sample of peripheral blood from a human subject; extracting mRNA molecules from the peripheral blood; amplifying a cDNA molecule encoding the PSA protein which comprises: (i) contacting the mRNA molecule extract with a nucleic acid molecule primer capable of specifically hybridizing to a nucleic acid sequence site included within the mRNA encoding the PSA protein under conditions allowing the primer to specifically

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hybridize to the mRNA encoding the PSA protein; (ii) contacting the composition of (i) with reverse transcriptase under conditions allowing the reverse transcriptase to transcribe a single stranded cDNA molecule copy of the mRNA molecule encoding the PSA protein; (iii) heat denaturing the cDNA and mRNA molecule of composition (ii); (iv) contacting the composition of (iii) with a first primer capable of specifically hybridizing to a first unique nucleic acid molecule sequence site located on the cDNA molecule complementary to the mRNA encoding the PSA protein under conditions allowing the first primer to specifically hybridize with the first unique nucleic acid molecule sequence site; (v) contacting the composition of (iv) with DNA polymerase under conditions allowing the DNA polymerase to synthesize a double stranded cDNA molecule encoding the PSA protein using the first primer to initiate synthesis and the single-stranded cDNA molecule as a template; (vi) heat denaturing the double-stranded cDNA molecule of composition (v); (vii) contacting the composition of (vi) with the first primer and a second nucleic acid molecule primer capable of specifically hybridizing with a second unique nucleic acid molecule sequence site wherein the second unique nucleic acid sequence site is located on the other cDNA strand 3' of the first unique site under conditions allowing the first and second primers to specifically hybridize with the first and second unique sequences, respectively, of the cDNA molecule encoding the PSA protein, and wherein the second primer comprises a nucleic acid molecule sequence of at least 15 nucleic acids that is complementary to a nucleic acid molecule sequence that is the same or substantially the same as the nucleic acid sequence shown in Figure 6; (viii) contacting the composition of (vii) with DNA polymerase under conditions allowing the DNA

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polymerase to synthesis cDNA molecules encoding the PSA protein using the first or second primers to initiate synthesis and the heat denatured cDNA molecule encoding the PSA protein as a template; and

5   (ix) repeating steps (vi) through (viii) for at least twenty five times; and detecting the presence of the cDNA molecule encoding the PSA protein and thereby determining the stage of prostate cancer.

10 —   This invention provides a method of determining the stage of prostate cancer in a human subject which comprises: collecting a sample of peripheral blood from a human subject; extracting mRNA molecules from the peripheral blood; amplifying a cDNA molecule

15   encoding the PSA protein which comprises: (i) contacting the mRNA molecule extract with a nucleic acid molecule primer capable of specifically hybridizing to a nucleic acid sequence site included within the mRNA encoding the PSA protein under

20   conditions allowing the primer to specifically hybridize to the mRNA encoding the PSA protein; (ii) contacting the composition of (i) with reverse transcriptase under conditions allowing the reverse transcriptase to transcribe a single stranded cDNA

25   molecule copy of the mRNA molecule encoding the PSA protein; (iii) heat denaturing the cDNA and mRNA molecule of composition (ii); (iv) contacting the composition of (iii) with a first synthesized oligonucleotide primer capable of specifically

30   hybridizing to a first unique nucleic acid molecule sequence site located on the cDNA molecule complementary to the mRNA encoding the PSA protein under conditions allowing the first primer to specifically hybridize with the first unique nucleic

35   acid molecule sequence site; (v) contacting the composition of (iv) with DNA polymerase under conditions allowing the DNA polymerase to synthesize

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a double stranded cDNA molecule encoding the PSA
protein using the first primer to initiate synthesis
and the single-stranded cDNA molecule as a template;
(vi) heat denaturing the double-stranded cDNA molecule
of composition (v); (vii) contacting the composition
of (vi) with the first primer and a second synthesized
oligonucleotide primer capable of specifically
hybridizing with a second unique nucleic acid molecule
sequence site wherein the second unique nucleic acid
sequence site is located on the other cDNA strand 3'
of the first unique site under conditions allowing the
first and second primers to specifically hybridize
with the first and second unique sequences,
respectively, of the cDNA molecule encoding the PSA
protein; (viii) contacting the composition of (vii)
with DNA polymerase under conditions allowing the DNA
polymerase to synthesis cDNA molecules encoding the
PSA protein using the first or second primers to
initiate synthesis and the heat denatured cDNA
molecule encoding the PSA protein as a template; and
(ix) repeating steps (vi) through (viii) for at least
twenty five times; and detecting the presence of the
cDNA molecule encoding the PSA protein and thereby
determining the stage of prostate cancer.

The preferred embodiment of the first synthesized
oligonucleotide primer is a synthesized
oligonucleotide primer comprising a nucleic acid
molecule with a nucleotide sequence of 5'-
GATGACTCCAGCCACGACCT-3'.

The preferred embodiment of the second synthesized
oligonucleotide primer is a synthesized
oligonucleotide primer comprising a nucleic acid
molecule with a nucleotide sequence of 5'-
CACAGACACCCCATCCTATC-3'.

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The most preferred embodiment of first and second nucleic acid molecule primers is a first primer with a nucleotide sequence of 5'-GATGACTCCAGCCACGACCT-3', and a second nucleic acid molecule primer with a nucleotide sequence of 5'-CACAGACACCCCATCCTATC-3', wherein the use of these two primers in the hereinabove disclosed methods for determining the stage of prostate cancer in a human subject produces an amplified cDNA which is 710 base pairs long.

This invention provides a method of determining the stage of prostate cancer in a human subject which comprises: collecting a sample of peripheral blood from a human subject; extracting mRNA molecules from the peripheral blood; amplifying a cDNA molecule encoding the PSA protein which comprises: (i) contacting the mRNA molecule extract with a nucleic acid molecule primer capable of specifically hybridizing to a nucleic acid sequence site included within the mRNA encoding the PSA protein under conditions allowing the primer to specifically hybridize to the mRNA encoding the PSA protein, wherein the primer capable of specifically hybridizing the mRNA encoding the PSA protein is an oligo$(dT)_N$ primer; (ii) contacting the composition of (i) with reverse transcriptase under conditions allowing the reverse transcriptase to transcribe a single stranded cDNA molecule copy of the mRNA molecule encoding the PSA protein; (iii) heat denaturing the cDNA and mRNA molecule of composition (ii); (iv) contacting the composition of (iii) with a first primer capable of specifically hybridizing to a first unique nucleic acid molecule sequence site located on the cDNA molecule complementary to the mRNA encoding the PSA protein under conditions allowing the first primer to specifically hybridize with the first unique nucleic acid molecule sequence site; (v) contacting the

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composition of (iv) with DNA polymerase under
conditions allowing the DNA polymerase to synthesize
a double stranded cDNA molecule encoding the PSA
protein using the first primer to initiate synthesis
5   and the single-stranded cDNA molecule as a template;
(vi) heat denaturing the double-stranded cDNA molecule
of composition (v); (vii) contacting the composition
of (vi) with the first primer and a second nucleic
acid molecule primer capable of specifically
10 --   hybridizing with a second unique nucleic acid molecule
sequence site wherein the second unique nucleic acid
sequence site is located on the other cDNA strand 3'
of the first unique site under conditions allowing the
first and second primers to specifically hybridize
15   with the first and second unique sequences,
respectively, of the cDNA molecule encoding the PSA
protein; (viii) contacting the composition of (vii)
with DNA polymerase under conditions allowing the DNA
polymerase to synthesis cDNA molecules encoding the
20   PSA protein using the first or second primers to
initiate synthesis and the heat denatured cDNA
molecule encoding the PSA protein as a template; and
(ix) repeating steps (vi) through (viii) for at least
twenty five times; and detecting the presence of the
25   cDNA molecule encoding the PSA protein and thereby
determining the stage of prostate cancer.

As used herein a oligo(dT)$_N$ primer is an
oligonucleotide primer consisting of sequence of
30   thymidine base pairs where N equals 12-18.

This invention provides a method of determining the
stage of prostate cancer in a human subject which
comprises: collecting a sample of peripheral blood
35   from a human subject, wherein the peripheral blood
contains PSA expressing cells at a concentration equal
to or greater than one PSA expressing cell per 100,000

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blood cells; extracting mRNA molecules from the peripheral blood; amplifying a cDNA molecule encoding the PSA protein which comprises: (i) contacting the mRNA molecule extract with a nucleic acid molecule primer capable of specifically hybridizing to a nucleic acid sequence site included within the mRNA encoding the PSA protein under conditions allowing the primer to specifically hybridize to the mRNA encoding the PSA protein; (ii) contacting the composition of (i) with reverse transcriptase under conditions allowing the reverse transcriptase to transcribe a single stranded cDNA molecule copy of the mRNA molecule encoding the PSA protein; (iii) heat denaturing the cDNA and mRNA molecule of composition (ii); (iv) contacting the composition of (iii) with a first primer capable of specifically hybridizing to a first unique nucleic acid molecule sequence site located on the cDNA molecule complementary to the mRNA encoding the PSA protein under conditions allowing the first primer to specifically hybridize with the first unique nucleic acid molecule sequence site; (v) contacting the composition of (iv) with DNA polymerase under conditions allowing the DNA polymerase to synthesize a double stranded cDNA molecule encoding the PSA protein using the first primer to initiate synthesis and the single-stranded cDNA molecule as a template; (vi) heat denaturing the double-stranded cDNA molecule of composition (v); (vii) contacting the composition of (vi) with the first primer and a second nucleic acid molecule primer capable of specifically hybridizing with a second unique nucleic acid molecule sequence site wherein the second unique nucleic acid sequence site is located on the other cDNA strand 3' of the first unique site under conditions allowing the first and second primers to specifically hybridize with the first and second unique sequences, respectively, of the cDNA molecule encoding the PSA

protein; (viii) contacting the composition of (vii) with DNA polymerase under conditions allowing the DNA polymerase to synthesis cDNA molecules encoding the PSA protein using the first or second primers to

5   initiate synthesis and the heat denatured cDNA molecule encoding the PSA protein as a template; and (ix) repeating steps (vi) through (viii) for at least twenty five times; and detecting the presence of the cDNA molecule encoding the PSA protein and thereby

10 —   determining the stage of prostate cancer.

The subject invention also provides a method for enhancing the detection of prostate specific antigen in a biological sample suspected of containing

15   prostate specific antigen which comprises:

(a)   extracting mRNA from the sample;

(b)   contacting the mRNA from step (a) with
20   reverse transcriptase under conditions
                allowing for the production of cDNA;

(c)   contacting the cDNA from step (b) with a
                pair of reverse transcriptase polymerase
25   chain reaction oligonucleotide primers
                capable of specifically hybridizing with DNA
                encoding prostate specific antigen, wherein
                one such primer is an oligonucleotide 12 to
                30 nucleotides in length and having the
30   sequence 5'-X-CACCCCATCCTA-Y-3'   and the
                second such primer is an oligonucleotide 12
                to 30 nucleotides in length and having the
                nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3'
                wherein each of X, X', Y, and Y' may be
35   present or absent, but if present X is A,
                GA,   AGA,   CAGA, ACAGA, CACAGA, ACACAGA,
                AACACAGA,   TAACACAGA,   ATAACACAGA,

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AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA;
X' is C, AC, GAC, TGAC, ATGAC, GATGAC,
TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or
CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT,
or TCTGTG; and Y' is C, CT, CTC, CTCA,
CTCAT, CTCATG, or CTCATGC; and wherein at
least one of the primers is covalently
linked to a suitably modified digoxigenin
molecule, under conditions allowing for
hybridization of both primers to any cDNA
encoding prostate specific antigen so as to
obtain a double stranded duplex comprising
the cDNA hybridized to the primers;

(d)   contacting the double stranded duplex from
        step (c) with DNA polymerase under
        conditions allowing for the extension of the
        primers to produce cDNA encoding prostate
        specific antigen and comprising a suitably
        modified digoxigenin molecule; and

(e)   detecting the suitably modified digoxigenin
        molecule from the cDNA from step (d) thereby
        detecting prostate specific antigen.

In a preferred embodiment of the above described
method the first primer is 5'-CACAGACACCCCATCCTATC-3'
and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

Those skilled in the art will recognize that steps (a)
and (b), extracting mRNA from a biological and
production of complementary DNA (cDNA) from the
extracted mRNA, respectively, as recited in the above
described method and the methods described
hereinbelow, are well known to those of ordinary skill
in the art. For an example of such methods and the
suitable conditions for carrying them out, see J.

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Sambrook, E.F. Fritsch, and T. Maniatis, Molecular
Cloning, A Laboratory Manual 2 ed. (Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, New York: 1989).

5   Those skilled in the art will recognize that the
methods of "contacting" recited in steps (c) and (d)
as well as the suitable "conditions" for carrying out
the steps of the above described method and the
methods described hereinbelow are readily determinable
10   — by those of ordinary skill in the art. Examples of
these methods and suitable conditions can be found in
PCR Protocols, A Guide to Methods and Applications,
Innis, M., Gelfand, D., Sninsky, J, and White, T.,
eds. (Academic Press, San Diego, CA: 1990). The term
15   "double stranded duplex" as used herein refers to the
composition of matter obtained when the primers have
specifically hybridized to the cDNA encoding prostate
specific antigen prior to the extension of the
primers.
20

In a preferred embodiment of the above described
method the detection in step (e) is accomplished using
a detectably labeled antibody which specifically binds
to the suitably modified digoxigenin molecule. As
25   used herein a "suitably modified digoxigenin molecule"
refers to the resulting chemical moeity which is
covalently attached to a molecule of interest when
labeling the molecule of interest with a digoxigenin
label. The digoxigenin label is thereafter used as a
30   means of detecting the molecule of interest by methods
of detecting the suitably modified digoxigenin
molecule. Such methods include, but are not limited
to use of antibodies which specifically bind to the
suitably modified digoxigenin molecule. Methods of
35   attaching a digoxigenin label, and, therefore,
covalently linking a suitably modified digoxigenin
molecule to a molecule of interest, are well known to

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those of ordinary skill in the art. An example of the use of digoxigenin labels is included in the Experimental Details below.

5   Suitable labels for antibodies to allow for their detection in assays of this type are known to those of skill in the art and include, but are not limited to enzymes, dyes, fluorescent markers such as fluorophores and chromaphores, colored bead,

10 -- radioactive isotope or biotin.

In a preferred embodiment the above described method the detectably labeled antibody is labeled with an enzyme. Examples of enzymes useful as labels for

15   detectably labeling antibodies include, but are not limited to, peroxidase, such as horseradish peroxidase, or other enzymes such as alkaline phosphatase and ß-galactosidase. In a particularly preferred embodiment of the subject invention the

20   enzyme is alkaline phosphatase.

In the practice of the above described method the biological sample can be taken from the peripheral blood or lymph nodes of a subject. Use of either type

25   of samples represents independently preferred embodiments of the subject invention.

The subject invention also provides a method for detecting prostate specific antigen in a biological

30   sample suspected of containing prostate specific antigen which comprises:

(a)   extracting mRNA from the sample;

35   (b)   contacting the mRNA from step (a) with reverse transcriptase under conditions allowing for the production of cDNA;

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(c)   contacting the cDNA from step (b) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(d)   contacting the double stranded duplex from step (c) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(e)   detecting the cDNA from step (d) thereby detecting prostate specific antigen.

In a preferred embodiment of the above described

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method the first primer is 5'-CACAGACACCCCATCCTATC-3'
and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

In one preferred embodiment the biological sample is
a sample taken from the peripheral blood of a subject.
In a second preferred embodiment the biological sample
is a sample from the lymph nodes of a subject.

The subject invention further provides a method of
diagnosing prostate cancer in a subject which
comprises:

(a) obtaining a biological sample from the
     subject;

(b) extracting mRNA from the sample;

(c) contacting the mRNA from (b) with reverse
     transcriptase under conditions allowing for
     the production of cDNA;

(d) contacting the cDNA from step (c) with a
     pair of reverse transcriptase polymerase
     chain reaction oligonucleotide primers
     capable of specifically hybridizing with DNA
     encoding prostate specific antigen, wherein
     one such primer is an oligonucleotide 12 to
     30 nucleotides in length and having the
     sequence 5'-X-CACCCCATCCTA-Y-3' and the
     second such primer is an oligonucleotide 12
     to 30 nucleotides in length and having the
     nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3'
     wherein each of X, X', Y, and Y' may be
     present or absent, but if present X is A,
     GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,
     AACACAGA, TAACACAGA, ATAACACAGA,
     AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA;

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X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(e)   contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f)   detecting the cDNA from step (e) thereby diagnosing prostate cancer in the subject.

In a preferred embodiment of the above described method the first primer is 5'-CACAGACACCCCATCCTATC-3' and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

In a preferred embodiment of the above described method at least one of the primers in the set of reverse transcriptase polymerase chain reaction primers is covalently linked to a suitably modified digoxigenin molecule.

In another preferred embodiment, the detection of cDNA in step (f) in the above described method comprises detection of the suitably modified digoxigenin molecule with a detectably labeled antibody which specifically binds to the suitably modified digoxigenin molecule.

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Suitable labels for antibodies to allow for their detection in assays of this type are known to those of skill in the art and include, but are not limited to enzymes, dyes, fluorescent markers such as fluorophores and chromaphores, colored bead, radioactive isotope or biotin.

In a preferred embodiment the above described method the detectably labeled antibody is labeled with an enzyme. Examples of enzymes useful as labels for detectably labeling antibodies include, but are not limited to, peroxidase, such as horseradish peroxidase, or other enzymes such as alkaline phosphatase and ß-galactosidase. In a particularly preferred embodiment of the subject invention the enzyme is alkaline phosphatase.

In one preferred embodiment of the above described method the biological sample is a sample taken from the peripheral blood of a subject. In a second preferred embodiment the biological sample is a sample from the lymph nodes of a subject.

The subject invention further provides a method of determining the stage of prostate cancer in a subject which comprises:

   (a)   obtaining a biological sample from the subject;

   (b)   extracting mRNA from the sample;

   (c)   contacting the mRNA from (b) with reverse transcriptase under conditions allowing for the production of cDNA;

   (d)   contacting the cDNA from step (c) with a

pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(e) contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f) detecting the cDNA from step (e) thereby determining the stage of prostate cancer in the subject.

In a preferred embodiment of the above described

method the first primer is 5'-CACAGACACCCCATCCTATC-3'
and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

In another preferred embodiment at least one of the primers in the pair of reverse transcriptase polymerase chain reaction oligonucleotide primers is covalently linked to a suitably modified digoxigenin molecule.

In a preferred embodiment of the above described method the detection of cDNA in step (f) comprises detection of the suitably modified digoxigenin molecule with a detectably labeled antibody which specifically binds to the suitably modified digoxigenin molecule.

Suitable labels for antibodies to allow for their detection in assays of this type are known to those of skill in the art and include, but are not limited to enzymes, dyes, fluorescent markers such as fluorophores and chromaphores, colored bead, radioactive isotope or biotin.

In a preferred embodiment the above described method the detectably labeled antibody is labeled with an enzyme. Examples of enzymes useful as labels for detectably labeling antibodies include, but are not limited to, peroxidase, such as horseradish peroxidase, or other enzymes such as alkaline phosphatase and ß-galactosidase. In a particularly preferred embodiment of the subject invention the enzyme is alkaline phosphatase.

In one preferred embodiment of the above described method the biological sample is a sample taken from the peripheral blood of a subject. In a second preferred embodiment the biological sample is a sample

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from the lymph nodes of a subject.

The subject invention also provides an oligonucleotide
12 to 30 nucleotides in length and having the sequence
5'-X-CACCCCATCCTA-Y-3' wherein each of X and Y may be
present or absent, but if present X is A, GA, AGA,
CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA,
ATAACACAGA,   AATAACACAGA,   AAATAACACAGA,   or
CAAATAACACAGA; and Y is T, TC, TCT, TCTG, TCTGT, or
TCTGTG.

In a preferred embodiment the oligonucleotide has the
nucleotide sequence 5'-CACAGACACCCCATCCTATC-3'.

In a preferred embodiment the oligonucleotide
described above is covalently linked to a suitably
modified digoxigenin molecule.

The subject invention further provides an
oligonucleotide 12 to 30 nucleotides in length and
having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-
3' wherein each of X' and Y' may be present or absent,
but if present X' is C, AC, GAC, TGAC, ATGAC, GATGAC,
TGATGAC,   GTGATGAC,   GGTGATGAC,   AGGTGATGAC   or
CAGGTGATGAC; and Y' is C, CT, CTC, CTCA, CTCAT,
CTCATG, or CTCATGC.

In a preferred embodiment the oligonucleotide has the
nucleotide sequence 5'-GATGACTCCAGCCACGACCT-3'.

In a preferred embodiment the oligonucleotide
described above is covalently linked to a suitably
modified digoxigenin molecule.

Finally, the subject invention provides a pair of
reverse transcriptase polymerase chain reaction
oligonucleotide primers for detecting the expression

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of the prostate specific antigen, wherein one such
primer is an oligonucleotide 12 to 30 nucleotides in
length and having the sequence 5'-X-CACCCCATCCTA-Y-3'
and the second such primer is an oligonucleotide 12 to
5   30 nucleotides in length and having the nucleotide
sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X,
X', Y, and Y' may be present or absent, but if present
X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,
AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA,
10   AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC,
TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC,
AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG,
TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT,
CTCATG, or CTCATGC.

15

In a preferred embodiment of the pair of reverse
transcriptase polymerase chain reaction
oligonucleotide primers described above the first such
primer is 5'-CACAGACACCCCATCCTATC-3' and the second
20   such primer is 5'-GATGACTCCAGCCACGACCT-3'.

In another preferred embodiment of the pair of reverse
transcriptase polymerase chain reaction
oligonucleotide primers described above at least one
25   of the primers is covalently linked to a suitably
modified digoxigenin molecule.

The reverse transcriptase polymerase chain reaction
oligonucleotide primers described above can be used as
30   the pair described immediately above or individually
with other primers known or later discovered by one of
ordinary skill in the art. Such uses can include use
of the primers as pimary sequences or as nested
primers to enhance specific amplification. Such uses
35   are contemplated to be within the scope of the claimed
invention.

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The following Experimental Details, and examples
contained therein, are set forth in an effort to
further explain the invention. The examples are not
intended to, and should not be interpreted to, limit
5   in any way the scope of the invention which is more
fully defined in the claims which follow thereafter.

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Experim ntail Details

Example 1 - Detection of Prostate Specific Antigen

I. Experimental Methods

A. Human Materials and Patient Selection

Two groups of prostate cancer patients were examined during our study; Group 1 comprised 10 patients with untreated metastatic prostate cancers (T4) confirmed by markedly elevated PSA values and the detection of metastatic lesions on a bone scan. Group 2 consisted of 48 patients with clinically localized prostate cancer (T1-T2c) who were scheduled for radical retropubic prostatectomy. All of these patients had received a digital rectal examination and a serum PSA determination (Hybritech Inc., San Diego, California) as well as a bone scan prior to surgery. In each case a CT scan and/or endorectal coil MRI was used for local staging of the tumor. Nine of the patients from group 2 received preoperative treatment with flutamide (750 mg/day) as a means to reduce bulky tumor mass prior to surgery. All physical examinations and surgical procedures for the patients in group 2 were performed by a single surgeon (CAO).

In addition to the prostate cancer patients, three groups of non-prostate cancer controls were analyzed: Group 1 comprised 20 females hospitalized at our institution, Group 2 consisted of 20 young males (ages 19-49) with no prior urological disease history, and Group 3 consisted of 25 age-matched males (ages 45-77, mean 62) that were under outpatient treatment for BPH. The age-matched control male patients all had serum PSA values less than 4.0 and negative digital rectal examinations. All patients and controls were

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evaluated at our institution, and their participation in this study was approved by the Internal Review Board.

B. Patient Samples

Venous blood (5cc) was collected from each individual in EDTA-treated collection tubes, placed immediately on ice, and processed within 3 hours of phlebotomy. For surgical candidates, the samples were obtained usually 1 week prior to surgery, at least two weeks from digital rectal examination and two weeks from prostate needle biopsy. Samples were diluted with an equal volume of phosphate-buffered saline (PBS) and carefully layered onto 8cc of Ficoll-Pack (Pharmacia Inc., Piscataway, NJ). The sample was centrifuged at 400 x g for 20 min., and the buffy coat cells were recovered. The cells were washed in 50 cc PBS and were re-centrifuged at 1000 x g for 30 min to produce a cell pellet.

A surgical specimen containing human prostate cancer was obtained from the human tumor bank operated by the Department of Pathology at this institution. This tissue was excised from a radical prostatectomy specimen and was frozen in liquid nitrogen and stored at -90°C.

C. Cell Culture

The human PSA-expression LnCaP cell line was originally established from a patient with metastatic prostate cancer to the pelvic lymph nodes (11). Monolayer propagation of these cells was in RPMI media supplemented with 5% Fetal Bovine Serum (FBS), 100 units/ml of penicillin and 0.1 mg/ml streptomycin. Every 2 days the cells received fresh medium and

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confluent cultures were dispersed with trypsin (0.05%):EDTA (0.02%) solution for subsequent passage. Serial dilutions of trypsinized cells were made in PBS to achieve cell densities as follows: 10,000 cells per ml, 1000 cells per ml, 100 cells per ml, and 100 cells per ml. These dilutions were added to 10^6 cultured immortalized B-cells (gift of R. Dalla Favera, Columbia University), centrifuged at 1500 rpm for 5 min., and prepared for RNA extraction.

D. RNA Extraction

Total RNA was obtained from these cells by a modification of the Guanididium thiocyanate/phenol/chloroform extraction technique (12) utilizing the RNAzole B reagent of Tel-Test, Inc., (Friendswood, Texas). The RNA pellet obtained after ethanol/sodium acetate precipitation was dried under vacuum and dissolved in 50μl of RNase-free water. Quantification of RNA was done based on spectrophotometric measurements at 260nm.

E. Reverse Transcription Reaction

An aliquot containing 1μg of total RNA was added to 0.5μg of oligo(dT)$_{12-18}$ primer (Gibco, BRL, Life Technologies, Inc.(, and brought to a final volume of 20 μl. The samples were placed at 65°C for 5 min., then chilled on ice for 5 min. The primer annealed RNA was added to 30 μl master reaction mixture so that the final concentration of the following components were achieved: 1mM of each dNTP, 50mM Tris-HCl (ph8.3), 75 mM KCl, 3mM MgCl$_2$, 10 mM DTT, 10 units per reaction of Placental RNase inhibitor, and 200 units per reaction of M-MuLV Reverse Transcriptase (Superscript II, Gibco BRL). The reaction was incubated at 42°C for 15 min. and the enzyme was heat

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inactivated at 95°C for 15 seconds.

F. Polymerase Chain Reaction (PCR)

5   Oligonucleotide primers, specific for the human PSA
cDNA coding region, were designed with the aid of the
primer analysis software, OLIGO (version 4.0, National
Biosciences, Plymouth, MN) and were synthesized by
national Biosciences. The 18-base pair primers were
10   —   designed to span across three exons; from exon 3 and
extending into exon 5 with the following sequences:
PSA3': 5'-CACAGACACCCCATCCTATC-3'
PSA5':5'-GATGACTCCAGCCACGACCT-3'
Oligonucleotide primers for human glyceraldehyde 3-
15   phosphate dehydrogenase (G3PDH) were obtained from
Clontech laboratories (Palo Alto, CA) and PCR for this
gene produce was performed in a separate reaction for
each sample. The PCR reaction was done in a total
volume of μl containing one fifth of the RT reaction
20   with a final concentration of the following reagents:
20mM Tris-HCl (ph 8.3 at 20°C), 50mM KCl, 1.5mM MgCl$_2$,
0.2mM of each dNTP, 2.5 units of Taq Polymerase and
10pmoles of each primer. When enhanced sensitivity
was desired, digoxigenin-11-deoxyUrdine Triphosphate
25   (Dig-dUTP) (Boehringer Mannheim Corp., Indianapolis,
IN) was added to a final concentration of 0.55μM. All
reactions were prepared with sets of PCR-dedicated
micropipettors using plugged tips to protect against
cross contamination.

30

A total of 35 cycles were completed with the following
specification: A) cycle 1, 95°C (4 min); B) cycles 2-
16, 95°C (1 min), 60°C (1 min), 72°C (1 min); D)
cycles 28-24, 95°C 1 min, 60°C 1 min., 72°C 2 min, and
35   the last cycle was at 72°C for 15 min. With the
addition of Dig-dUTP in the PCR reaction the number of
cycles was decreased to 25 with the removal of cycle

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set C. The PCR products were stored at 4°C until electrophoresis. Aliquots of the reaction were electrophoresed on 2.5% agarose gels in TEA buffer at 80V. The gels were stained with ethidium bromide and were viewed under UV light.

G. Cloning of the PSA-PCR Product

The 710 base pair band representing the PCR produce derived from a human prostate tumor was excised and purified from a 2.0% agarose gel. This PCR product was ligated into the PCR cloning site of the pCR II vector (Invitrogen Corp.,(. The cloned insert was sequenced using SP6 and T7 primers by dideoxy-nucleotide sequence analysis (13). The sequence obtained for this DNA insert was compared with the Human PSA genomic sequence of Klobeck, et al. (14) obtained in the Genebank (Accession # X14810).

H. Enhanced PCR Detection

Digoxigenin-enhanced detection of the PSA PCR reaction product was accomplished with the use of Genius DNA labeling kit (Boehringer Mannhein) with modifications. Southern blotting of the gel fractionated PCR reaction onto positively charged nylon membranes (Boehringer Mannheim) was accomplished following standard techniques (15) and was fixed onto the membrane by UV exposure. Membrane washing and blocking was according the manufacture's specifications. Alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragment) was used at a 1:50,000 dilution in Genius 2 buffer (2% blocking agent in 100mM Tris-HCl (ph 7.5 at 20°C), 150mM NaCl prior to incubation with the membrane. The chemiluminescent substrate, Lumi-Phos 530 [0.33mM Lumigen PPD [4-methoxy-4-(3-phosphate-phenyl)-sprio(1,2-dioxetane-3,2-adamantane) disodium

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salt]; 0.88 mM MgCL$_2$; 1.13mM cetyltrimethy-ammonium bromide; 0.035mM fluorescein surfactant], was diluted 1:1 in 100mMTris-HCL (pH 9.5 at 20°C), 100mM NaCl, 50mM MgCL$_2$ and a small amount was carefully spread across the membrane. The wrapped membrane was exposed to X-ray film, typically, for only several minutes and the film was developed.

I. Statistical Methods

Logistic regression analysis was used to determine the statistical significance of each of the following preoperative factors: PSA, PSAD, imaging (CT-scan and/or endorectal MRI), and PCR. A Chi-square score was determined for each independent variable to evaluate the likelihood of predicting margin positive-disease. In addition, preoperative factors were examined with and without the PCR assay to determine if deletion or addition of assay results would add to the prediction of margin positive disease.

II. Results

A. Detection of PSA-Synthesizing Cells by PCR

RNA was extracted from the LNCaP cell line and from a surgically-removed specimen containing prostate cancer. This RNA was reverse transcribed to cDNA and then utilized in a PCR reaction with primers designed to amplify a 710 bp DNA fragment from human PSA RNA. Following electrophoresis of the PCR products, we were able to identify the appropriate DNA fragment corresponding to PSA only in reactions containing cDNA (Figure 1). This 710 bp fragment was cloned into a plasmid vector and was sequenced. The sequence of the insert (not shown here) corresponded at every nucleotide to the sequence of human PSA appearing in

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the genebank.

B. PCR of LnCaP Dilution

By diluting known quantities of LnCaP cells into
cultured B-lymphocytes, were able to evaluate the
minimum number of PSA-synthesizing cells detectable by
PCR> Serial dilutions of LNCaP cells were added to
aliquots containing 10^6 human B-lymphocytes. RNA was
extracted from these diluted specimens and was assayed
in individual reactions by RT-PCR. A portion of the
reaction product was electrophoresed on an agarose gel
and, as shown in Figure 2, when the reaction included
primers for human PSA, we could detect as few as 10
PSA-synthesizing cells (1 in 100,000). Duplicate
aliquots of cDNA from each of these specimens were
also run for PCR using primers for human G3PDH, and,
in contrast to the reactions with human PSA primers,
in which the sample lacking LNCaP cells failed to give
a reaction product, all specimens yielded the expected
983 bp G3PDH DNA fragment (Figure 2). All subsequent
reactions on patient specimens were carried out so
that each cDNA had a duplicate reaction containing
G3PDH primers as a positive control for cDNA
integrity. Each subsequent PCR experiment involving
patient samples included one specimen of LNCaP cDNA to
serve as a positive control reaction for PSA
detection. In addition, a sample having no added cDNA
was included among the specimen set to provide a
negative control and to ensure against contamination
of PCR reagents.

C. PCR analysis on Bloods From Control Patients

PCR analysis was performed on the RNA extracted from
venous blood lymphocytes obtained from 20 male
subjects (ages 19-41), and 20 female patients (ages

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23-76). None of these control specimens revealed a positive PCR reaction (Fig. 3 and Fig. 4). In addition, lymphocyte fraction RNA of 25 age-matched BPH patients were analyzed to determine the possible

5   effect of BPH on the PCR assay. None of these patients revealed a positive PCR fragment as assessed by ethidium bromide staining of the agarose gel following electrophoresis. The addition of digoxigenin-dUTP to the PCR reaction enabled us to

10   increase the sensitivity of detection of the PSA-PCR fragment as described in more detail for our prostate cancer patients. The enhanced method of detection was performed on each of these groups of patients, and the 710 bp PSA DNA fragment was not detected in any

15   specimen (not shown).

D. PCR analysis of Bloods from Metastatic Prostate Cancer Patients

20   Ten patients with confirmed but untreated metastatic prostate cancer were analyzed by the RT-PCR assay. All patients in this category had positive bone scans indicating the presence of bony metastases. PSA values for patients in this group ranged from 42 to

25   412 ng/ml. The peripheral lymphocyte RNA RT-PCR assay for 8 of these 10 patients revealed the presence of the 710 bp PSA PCR fragment. Five of these positives were revealed by ethidium bromide visualization and 3 additional specimens were revealed by the enhanced

30   method for detection (not shown here).

E. PCR Analysis on Bloods from Localized Prostate Cancer Patients

35   Lymphocyte fraction RNAs from a total of 48 patients with clinically confined prostate cancer were analyzed by RT-PCR for PSA. Only 4 of these patients were

positive for the PSA-PCR reaction by ethidium bromide staining analysis following agarose gel electrophoresis. However, an additional 7 patients were found to be positive with the use of the enhanced method of detection (Figure 5, A and B), bringing the total to 11 (or 22.9% of patients in this category). The radical prostatectomy specimens obtained from these patients were subsequently analyzed as to the status of the Gleason grade of the tumor; whether the tumor extended into the prostatic capsule (capsular penetration); the presence of tumor at the surgical margins; and seminal vesicle involvement. None of these patients had detectable metastasis to the pelvic lymph nodes.

Table 1 summarizes the correlation of the patient PCR analysis with respect to pathological tumor stage. In the 48 patients operated for clinically localized prostate cancer, 15 (31.2%) were subsequently found to have tumor penetrating the prostatic capsule. Of these 15 patients, an RT-PCR fragment for PSA was amplified from 11 of the blood specimens. Therefore, if the RT-PCR blood assay was utilized as a means to discriminate prostate cancer patients with only capsular invasion from this population, it would have a sensitivity of 73% and a specificity of 89%.

Ten of the 48 patients were subsequently found to have tumor present at the surgical margin (20.8% of surgical patient population). Nine of these 10 margin positive patients were also positive for PSA by RT_PCR> We calculated the sensitivity and specificity of the PCR assay for detecting margin positive disease in this patient population at 90% and 94%, respectively. Based on our statistical analysis, the RT-PCR assay is a

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Tabl 1. Comparison of PCR findings in 48 patients with clinically localized prostate cancers and final pathological stage. Sensitivity, specificity, and predictive values were calculated for PCR in each category of pathology.

	POSITIVE MARGINS	CAPSULAR PENETRATION	POSITIVE SEMINAL VESICLE
Number of Patients	10/48 (20.8%)	15/48 (31.2%)	2/48 (4.16%)
PCR+	9	11	1
Sensitivity	90%	73%	50%
Specificity	94%	89.2%	75.6%
Positive Predictive Value	82%	78%	78%
Negative Predictive Value	93%	61%	18%

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strong predictor of tumor spread through and beyond the prostatic capsule. An odds ration analysis of this 48 patient sample (patient group 2) estimating the likelihood that the presence of tumor at the surgical margin would be associated with a positive PCR assay was 162 (95% confidence limits: 13.1 to 1991.6), respectively. Finally, 2(4.16%) of the 48 surgical patients were found to have tumor invading the seminal vesicle. The PCR assay had a 50% sensitivity and 75.6% specificity for detecting patients with disease into the seminal vesicles. However, there were only 2 patients in the study with positive seminal vesicles, therefore, no good statistical correlation could be determined for this subgroup of patients. Used as an independent variable to predict prostate cancer confined to the prostate gland, RT-PCR for PSA had a positive predictive value of 81.8%.

The staging results predicted by preoperative imaging modalities were statistically compared to the results obtained with PCR. Prior to surgery, all patients had a serum PSA obtained and DRE by performed by one examiner (CAO). Patients either had a CT-scan of the pelvis, an endorectal coil MRI, or both to detect extraprostatic disease. Imaging sensitivity and specificity for disease confined to the prostate is listed in Table 2 and compared to the results of the RE-PCR assay. RT-PCR revealed the highest overall sensitivity for detecting extraporstatic tumor (73%).

Additionally, a stepwise logistic regression analysis was performed to identify the single variable (PSA, PSAD, CT scan, endorectal MRI or PCR) which would predict cancer at the surgical margin. The RT-PCR assay had the highest Chi-square score (χ^2=25.3 p>0.0001) for this distinction (Table 2). In fact, RT-PCR proved to be the only variable which met a

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Table 2. Comparison of PCR to standard pre-operative staging modalities in 48 patients undergoing radical prostatectomy. PCR was found to have the highest sensitivity and predictor of confined prostate cancer (negative predictive value).

	PCR	DRE	CT SCAN	ENDORECTAL COIL MRI
TRUE (+)	11	2	2	1
TRUE (−)	32	37	28	3
FALSE (+)	0	0	5	1
FALSE (−)	5	9	6	3
SENSITIVITY TP/TP + FN	68.7%	18%	25%	25%
SPECIFICITY TN/TN + FP	100%	100%	84.8%	75%

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confidence level of 95%. Prostate specific antigen
density (PSAD) is defined as a unitless ratio combining
the value of the serum PSA measurement with the
prostate volume. PSAD has been previously shown to be
5   useful predictor for distinguishing benign from
malignant prostate tissue (16). The mean PSAD was 0.29
in our surgical patient population with a standard
deviation of 0.25. Table 3 compares the PSAD to the
pathological stage for these patients. In this series,
10 —   PSAD as a single predictor of margin positive disease
was second best to RT-PCR. Stepwise logarithmic
analysis of PSAD for this group of patients revealed a
Chi-square score of 11.0, p>.0009. Simple serum PSA
values are compared to the final pathological stage in
15   Table 4. The majority of patients (54%) had PSA values
in the 4 to 10 ng/ml range. The mean PSA value for all
patients was 11.2 ng/ml (S.D. ±7.9). The Chi-square
score for PSA as a single-modality predictor of
extraprostatic disease was calculated to be
20      $\chi^2=7.09$p>0.0001. Only 2 patients had PSA values
greater than 30. Both of these patients were found to
have positive surgical margins and were also PCR
positive.

25   It is of further interest that 9 of the 48 radical
prostatectomy patients had received flutamide therapy
prior to their surgery and prior to the phlebotomy from
which the PSA-PCR assay was performed. This subset of
patients included 2 which had a positive PCR reaction
30   and 7 which were negative. Cochran-Mantel-Haenszel
summary odds ratio analysis controlling for patients
given flutamide therapy resulted in an adjusted odds
ration of 69.7 (95% confidence limits: 6.6 to 725.6).
Furthermore, removal of these flutamide-treated
35   patients from the rest of the surgical patients did not
affect the strong correlation between positivity on the
PCR assay and the presence of tumor at the surgical

Table 3. Correlation of PSAD and pathological stage in 48 patients with clinically localized cancer of the prostate.

PSAD	NUMBER OF PATIENTS	+PCR	POSITIVE MARGINS	POSITIVE CAPSULE	POSITIVE SEMINAL VESICLE
0-0.15	10	1(10%)	1(10%)	1(10%)	0(0%)
0.16-0.30	20	3(15%)	2(10%)	7(35%)	0(0%)
0.31-0.60	10	3(30%)	2(20%)	2(20%)	1(10%)
>0.60	8	4(50%)	5(63%)	6(75%)	1(12.5%)
TOTAL	48(100%)	11(22.9%)	10(20.8%)	15(31.2%)	2(4.2%)

Table 4. Correlation of serum PSA and pathological stage in 48 patients with clinically localized cancer of the prostate.

PSA/(ng/ml)	NUMBER OF PATIENTS	+PCR	POSITIVE MARGINS	POSITIVE CAPSULE	POSITIVE SEMINAL VESICLE
0-4.0	2	0(0%)	0(0%)	0(0%)	0(0%)
4.1-10.0	26	3(11.5%)	3(11.5%)	6(23%)	0(0%)
10.1-20	14	3(21.4%)	3(21.4%)	6(42.8%)	0(0%)
20.1-30	4	3(75%)	2(50%)	2(50%)	2(50%)
30.1-40	2	2(100%)	2(100%)	2(100%)	0(0%)
Total	48	11	10	16	2

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margin. With this change, the RT-PCR assay now had 100% sensitivity for identifying margin positive disease with a specificity of 93.5%.

5   **III. Discussion**

In this study, we have determined that the detection of blood-borne PSA-synthesizing cells by RT-PCR can be accomplished in patients with localized as well as
10 --   metastatic prostate cancer and this detection provides a reliable marker for predicting local invasion of a prostate tumor prior to surgical procedures. PSA, a 34,000 kd glycoprotein, is a prostate-specific serine protease which is expressed exclusively by prostate
15   epithelial cells, the cells most frequently involved in prostatic oncogenesis (17). In recent years, assays used to detect this protein in the blood have revolutionized the management of prostate cancer patients by allowing the earlier detection of prostate
20   tumors as well as by providing a more effective means to follow the progression of the disease. As we have shown here, the specificity of PSA for prostate cells has further enabled the development of this RT-PCR based assay that can detect as few as 1 PSA-
25   synthesizing cell in 100,000 blood cells. When this assay was applied to peripheral blood specimens taken from prostate cancer patients with confirmed metastases, it was positive for the overwhelming majority (80%) of patients in this category available
30   for our study. The biological significance of these prostate cells in the circulation is not known at the present time. However, the high percentage of frank metastatic patients that give a positive reaction on this assay suggests that it is detecting circulating
35   metastatic prostate cells. When this assay was applied to blood specimens taken from prostate cancer patients with suspected prostate-confined disease, it was

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positive only in a much smaller subset (22.9%) of these patients. Because the subset of patients detected by the PSA-PCR assay correlated strongly with local invasive spread of the cancer, the significance of this determination may lie in a more accurate measure of staging than with the imaging modalities utilized today (ie. MRI, bone scan and CT-scan). Statistical analysis of the data gathered in this survey supports the presumption that patients with localized prostate cancer that react positively on this assay represent a subset that are at increased risk for recurrent disease, despite surgical extirpation of the gland.

Two previous studies in the literature have addressed the concept of hematogenous dissemination of prostate cancer. Using flow cytometry, Hamdy et al. demonstrated that PSA-positive cells could be detected in 25 prostate cancer patients with bone metastasis while none of the control patients had PSA-positive cells in the peripheral blood (18). The results of this study were peculiar in the some patients had greater than 50% PSA-positive cells in the circulating lymphocyte fraction. However, a second report, describing the PCR amplification of a PSA DNA fragment from reverse transcribed peripheral blood RNA of metastatic prostate cancer patients confirmed its detection in 4 of 12 patients (33%) whereas it was never identified in a small group of control (non-cancer) patients (10). Our current investigation included 3 groups of non-cancer control patients and we never detected an appropriate PSA RT-PCR fragment from any blood specimen obtained from women, young males, or age-matched control males with benign enlargement of the prostate gland (total of 65 specimens). Therefore, as supported by the previous study (10) and by our current results, there is no evidence to date that non-prostate cancer patients have PSA-synthesizing cells

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present in the peripheral circulation. In contrast, untreated metastatic prostate cancer patients were overwhelmingly positive on this assay (80%). Moreover, the only other group that gave a positive reaction on this assay were a minority of patients (11 of 48 or 22.9%) with suspected clinically localized prostate cancer. Therefore, our results indicate that the blood-based RT-PCR assay for PSA is specific for prostate cancer patients.

In our series of patients with localized prostate cancer, 10 patients were found to have tumor at the surgical margin subsequent to their radical surgery. Of these 10 patients, 9 had a positive RT-PCR reaction for PSA (90% sensitivity). Of the remaining 38 patients with margin negative disease, only 2 were found to have a positive PCR result (94% specificity). This is convincing evidence that a strong clinical correlation exists between the local extent of the tumor and hematogenous dissemination. In the patients with capsular penetration, the PCR assay was less sensitive than for margin positive disease (73%). Prostate cell dissemination into the circulation may therefore be a factor of the primary tumor volume. As tumor volume increases and encroaches on blood vessels, the basement membrane of endothelium may break down, allowing for tumor cells to enter the circulation. Interestingly, none of the patients in this group were found to have lymph node involvement, and all patients had negative bone scans, indicating that hematogenous dissemination may occur prior to lymphatic or bony involvement.

In this study, a fraction of the patients were given a course of flutamide therapy for "downstaging" of bulky tumors. Since 2 of these treated patients were positive on the PCR assay, it is clear that a short

course of flutamide therapy does not abolish the ability of this PCR assay to detect circulating prostate cells. Considering that the only patient that had tumor at the surgical margin and remained unreactive on the assay was of this treatment group, allows for the possibility that flutamide treatment prior to phlebotomy may decrease the sensitivity of the PCR assay. However, the results of the Cochran-Mantel-Haenzszel summary odds ratio analysis controlling for flutamide-treated patients remained statistically significant, suggesting that flutamide does not meaningfully alter the sensitivity of the PSA-PCR assay.

The presence or absence of extracapsular prostate cancer is often the critical factor in therapeutic decisions regarding prostate cancer. Radical prostatectomy has been shown to be the most effective therapy when disease remains confined to the gland (19, 20). Microscopic disease that invades into the capsule or to the surgical margin significantly reduces the chance for cure. Several studies have shown that tumor can be detected at the surgical margin in 11 to 32% of clinical stage A patients and 23-71% of clinical stage B prostate cancer patients (21, 22). These high numbers directly reflect the inaccuracies of the current preoperative staging modalities. A recent report from a multi-institutional investigation comparing MRI and TRUS in the early staging of prostate cancer concluded that these modalities are not highly accurate because neither technique could identify microscopic spread (23). Eight patients in this current study had MRI with endorectal coils prior to surgery. This expensive modality was able to predict confined tumor in 50% of the cases. If the MRI was interpreted as positive, i.e. extracapsular, the MRI results were only 50% accurate. From a statistical

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standpoint, when PCR was compared to preoperative
imaging, the PCR assay had the highest reliability
(based on Chi-square analysis) in predicting margin
positive disease.

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The most common used imaging modality for local staging
cancer is still the CT-scan, even though this modality
traditionally understages a significant number of
patients. From the perspective of prognostic ability
10   and cost effectiveness perspective CT scanning is under
serious investigation (24). Review of the CT-scan
findings from our study do not contrast with those
reported in other series (25,26). The CT-scan revealed
a 25% sensitivity, and could accurately predict disease
15   outside of the gland in only 28.5% of the cases.

Other studies in the literature have correlated the
serum PSA and PSAD with extraprostatic involvement and
lymph node metastasis (27, 28, 29, 30). In the 48
20   patients with localized prostate cancer, the PSAD for
patients with positive and negative results on RT-PCR
was 0.47 and 0.25 respectively. Recent investigations
have demonstrated that a PSAD value greater than 0.3 is
associated with a high incidence of extraprostatic
25   disease (31). Our findings of PSA-synthesizing cells
in the peripheral circulation of patients circulation
of patients with localized prostate cancer and this
high PSAD are consistent with this hypothesis.

30   To our knowledge, this is also the first report
employing digoxigenin-labeling as a detection system to
enhance the PCR reaction. This enhanced method of
detection enabled us to detect an additional 7 patients
with localized prostate cancer that would have been
35   missed by conventional ethidium-bromide staining of the
agarose gel. We also performed the enhanced detection
on all control patient reactions to ensure that these

were negative by both techniques, and found that all controls proved negative.

The possibility of staging prostate cancer patients with a 5cc sample of peripheral blood has several important implications. The future use of such a PCR-based assay has the potential for significantly decreasing the total cost of prostate cancer management. Since this assay appears to be more accurate than current screening modalities, it may enable a reduction not only in the number of preoperative CT-scans and MRI's, but also in the number of radical cancer operations performed. The social and ethical considerations extend to the patient with prostate cancer who may be spared the morbidity of an operation that would otherwise be indicated. And finally, our ability to predict the cure rate in patients undergoing radical cancer surgery will be greatly enhanced with this highly sensitive, accurate test.

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30

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35

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in patients with prostate cancer.   J. Urol.
142:1011-1017 (1989).

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       prostate-specific   antigen,   and   transrectal
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10 -- M.C.   The use of PSAD to predict D-0 prostatic
       carcinoma.   J. Urol. 149:300A (1993).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Katz, Aaron E.; Buttyan, Ralph; Raffo, Anthony;
            Olsson, Carl A.

   (ii) TITLE OF INVENTION: A Method For Molecular Staging Of
                Prostate Cancer

  (iii) NUMBER OF SEQUENCES: 4

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       (F) ZIP: 10036

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       (A) MEDIUM TYPE: Floppy disk
       (B) COMPUTER: IBM PC compatible
       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
       (A) APPLICATION NUMBER:
       (B) FILING DATE:
       (C) CLASSIFICATION:

 (viii) ATTORNEY/AGENT INFORMATION:
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       (B) REGISTRATION NUMBER: 28,678
       (C) REFERENCE/DOCKET NUMBER: 43677-A-PCT/JPW/MJG

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       (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1729 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (vi) ORIGINAL SOURCE:
       (A) ORGANISM: Homo sapiens

   (ix) FEATURE:
       (A) NAME/KEY: CDS
       (B) LOCATION: 378..1088

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGTTTCCCT TCTCCCAGTC CAAGACCCCA AATCACCACA AAGGACCCAA TCCCCAGACT   60

CAAGATATGG TCTGGGCGCT GTCTTGTGTC TCCTACCCTG ATCCCTGGGT TCAACTCTGC   120

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```
TCCCAGAGCA TGAAGCCTCT CCACCAGCAC CAGCCACCAA CCTGCAAACC TAGGGAAGAT      180

TGACAGAATT CCCAGCCTTT CCCAGCTCCC CCTGCCCATG TCCCAGGACT CCCAGCCTTG      240

GTTCTCTGCC CCCGTGTCTT TTCAAACCCA CATCCTAAAT CCATCTCCTA TCCGAGTCCC      300

CCAGTTCCTC CTGTCAACCC TGATTCCCCT GATCTAGCAC CCCCTCTGCA GGTGCTGCAC      360

CCCTCATCCT GTCTCGG ATT GTG GGA GGC TGG GAG TGC GAG AAG CAT TCC          410
                    Ile Val Gly Gly Trp Glu Cys Glu Lys His Ser
                     1               5                   10

CAA CCC TGG CAG GTG CTT GTA GCC TCT CGT GGC AGG GCA GTC TGC GGC          458
Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly Arg Ala Val Cys Gly
            15              20                  25

GGT GTT CTG GTG CAC CCC CAG TGG GTC CTC ACA GCT ACC CAC TGC ATC          506
Gly Val Leu Val His Pro Gln Trp Val Leu Thr Ala Thr His Cys Ile
        30                  35                  40

AGG AAC AAA AGC GTG ATC TTG CTG GGT CGG CAC AGC CTG TTT CAT CCT          554
Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His Ser Leu Phe His Pro
    45                  50                  55

GAA GAC ACA GGC CAG GTA TTT CAG GTC AGC CAC AGC TTC CCA CAC CCG          602
Glu Asp Thr Gly Gln Val Phe Gln Val Ser His Ser Phe Pro His Pro
60                  65                  70                  75

CTC TAC GAT ATG AGC CTC CTG AAG AAT CGA TTC CTC AGG CCA GGT GAT          650
Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe Leu Arg Pro Gly Asp
                80                  85                  90
```

```
GAC TCC AGC CAC GAC CTC ATG CTG CTC CGC CTG TCA GAG CCT GCC GAG          698
Asp Ser Ser His Asp Leu Met Leu Leu Arg Leu Ser Glu Pro Ala Glu
                95                  100                 105

CTC ACG GAT GCT ATG AAG GTC ATG GAC CTG CCC ACC CAG GAG CCA GCA          746
Leu Thr Asp Ala Met Lys Val Met Asp Leu Pro Thr Gln Glu Pro Ala
            110                 115                 120

CTG GGG ACC ACC TGC TAC GCC TCA GGC TGG GGC AGC ATT GAA CCA GAG          794
Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly Ser Ile Glu Pro Glu
        125                 130                 135

GAG TTC TTG ACC CCA AAG AAA CTT CAG TGT GTG GAC CTC CAT GTT ATT          842
Glu Phe Leu Thr Pro Lys Lys Leu Gln Cys Val Asp Leu His Val Ile
140                 145                 150                 155

TCC AAT GAC GTG TGT GCG CAA GTT CAC CCT CAG AAG GTG ACC AAG TTC          890
Ser Asn Asp Val Cys Ala Gln Val His Pro Gln Lys Val Thr Lys Phe
                160                 165                 170

ATG CTG TGT GCT GGA CGC TGG ACA GGG GGC AAA AGC ACC TGC TCG GGT          938
Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys Ser Thr Cys Ser Gly
            175                 180                 185

GAT TCT GGG GGC CCA CTT GTC TGT AAT GGT GTG CTT CAA GGT ATC ACG          986
Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val Leu Gln Gly Ile Thr
        190                 195                 200

TCA TGG GGC AGT GAA CCA TGT GCC CTG CCC GAA AGG CCT TCC CTG TAC          1034
Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu Arg Pro Ser Leu Tyr
        205                 210                 215

ACC AAG GTG GTG CAT TAC CGG AAG TGG ATC AAG GAC ACC ATC GTG GCC          1082
Thr Lys Val Val His Tyr Arg Lys Trp Ile Lys Asp Thr Ile Val Ala
220                 225                 230                 235
```

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```
AAC CCC TGAGCACCCC TATCAACTCC CTATTGTAGT AAACTTGGAA CCTTGGAAAT      1138
Asn Pro
```

```
GACCAGGCCA AGACTCAGGC CTCCCCAGTT CTACTGACCT TTGTCCTTAG GTGTGAGGTC      1198
```

```
CAGGGTTGCT AGGAAAAGAA ATCAGCAGAC ACAGGTGTAG ACCAGAGTGT TTCTTAAATG      1258
```

```
GTGTAATTTT GTCCTCTCTG TGTCCTGGGG AATACTGGCC ATGCCTGGAG ACATATCACT      1318
```

```
CAATTTCTCT GAGGACACAG ATAGGATGGG GTGTCTGTGT TATTTGTGGG GTACAGAGAT      1378
```

```
GAAAGAGGGG TGGGATCCAC ACTGAGAGAG TGGAGAGTGA CATGTGCTGG ACACTGTCCA      1438
```

```
TGAAGCACTG AGCAGAAGCT GGAGGCACAA CGCACCAGAC ACTCACAGCA AGGATGGAGC      1498
```

```
TGAAAACATA ACCCACTCTG TCCTGGAGGC ACTGGGAAGC CTAGAGAAGG CTGTGAACCA      1558
```

```
AGGAGGGAGG GTCTTCCTTT GGCATGGGAT GGGGATGAAG TAAGGAGAGG GACTGACCCC      1618
```

```
CTGGAAGCTG ATTCACTATG GGGGGAGGTG TATTGAAGTC CTCCAGACAA CCCTCAGATT      1678
```

```
TGATGATTTC CTAGTAGAAC TCACAGAAAT AAAGAGCTGT TATACTGTGA A      1729
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 237 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Ile Val Gly Gly Trp Glu Cys Glu Lys His Ser Gln Pro Trp Gln Val
1               5               10              15

Leu Val Ala Ser Arg Gly Arg Ala Val Cys Gly Gly Val Leu Val His
            20              25              30

Pro Gln Trp Val Leu Thr Ala Thr His Cys Ile Arg Asn Lys Ser Val
        35              40              45

Ile Leu Leu Gly Arg His Ser Leu Phe His Pro Glu Asp Thr Gly Gln
    50              55              60

Val Phe Gln Val Ser His Ser Phe Pro His Pro Leu Tyr Asp Met Ser
65              70              75              80

Leu Leu Lys Asn Arg Phe Leu Arg Pro Gly Asp Asp Ser Ser His Asp
                85              90              95

Leu Met Leu Leu Arg Leu Ser Glu Pro Ala Glu Leu Thr Asp Ala Met
            100             105             110

Lys Val Met Asp Leu Pro Thr Gln Glu Pro Ala Leu Gly Thr Thr Cys
        115             120             125

Tyr Ala Ser Gly Trp Gly Ser Ile Glu Pro Glu Glu Phe Leu Thr Pro
    130             135             140

Lys Lys Leu Gln Cys Val Asp Leu His Val Ile Ser Asn Asp Val Cys
145             150             155             160

Ala Gln Val His Pro Gln Lys Val Thr Lys Phe Met Leu Cys Ala Gly
```

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```
                    165                  170                    175
Arg Trp Thr Gly Gly Lys Ser Thr Cys Ser Gly Asp Ser Gly Gly Pro
            180                  185                    190
Leu Val Cys Asn Gly Val Leu Gln Gly Ile Thr Ser Trp Gly Ser Glu
            195                  200                    205
Pro Cys Ala Leu Pro Glu Arg Pro Ser Leu Tyr Thr Lys Val Val His
    210                  215                    220
Tyr Arg Lys Trp Ile Lys Asp Thr Ile Val Ala Asn Pro
225                  230                    235
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Homo sapiens

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CACAGACACC CCATCCTATC   20

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Homo sapiens

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATGACTCCA GCCACGACCT   20

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What is claimed is:

1.   A method for enhancing the detection of prostate
specific antigen in a biological sample suspected of
containing prostate specific antigen which comprises:

(a)   extracting mRNA from the sample;

(b)   contacting the mRNA from step (a) with
        reverse transcriptase under conditions
        allowing for the production of cDNA;

(c)   contacting the cDNA from step (b) with a
        pair of reverse transcriptase polymerase
        chain reaction oligonucleotide primers
        capable of specifically hybridizing with DNA
        encoding prostate specific antigen, wherein
        one such primer is an oligonucleotide 12 to
        30 nucleotides in length and having the
        sequence 5'-X-CACCCCATCCTA-Y-3'   and the
        second such primer is an oligonucleotide 12
        to 30 nucleotides in length and having the
        nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3'
        wherein each of X, X', Y, and Y' may be
        present or absent, but if present X is A,
        GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,
        AACACAGA, TAACACAGA, ATAACACAGA,
        AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA;
        X' is C, AC, GAC, TGAC, ATGAC, GATGAC,
        TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or
        CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT,
        or TCTGTG; and Y' is C, CT, CTC, CTCA,
        CTCAT, CTCATG, or CTCATGC; and wherein at
        least one of the primers is covalently
        linked to a suitably modified digoxigenin
        molecule, under conditions allowing for
        hybridization of both primers to any cDNA

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encoding prostate specific antigen so as to
obtain a double stranded duplex comprising
the cDNA hybridized to the primers;

5   (d)   contacting the double stranded duplex from
step (c) with DNA polymerase under
conditions allowing for the extension of the
primers to produce cDNA encoding prostate
specific antigen and comprising a suitably
10   modified digoxigenin molecule; and

(e)   detecting the suitably modified digoxigenin
molecule from the cDNA from step (d) thereby
detecting prostate specific antigen.

15

2.   The method of claim 1, wherein in step (c) the
first primer is 5'-CACAGACACCCCATCCTATC-3' and the
second primer is 5'-GATGACTCCAGCCACGACCT-3'.

20   3.   The method of claim 1, wherein in step (e)
detection is accomplished using a detectably labeled
antibody which specifically binds to the suitably
modified digoxigenin molecule.

25   4.   The method of claim 3, wherein the detectably
labeled antibody is labeled with an enzyme.

5.   The method of claim 4, wherein the enzyme is
alkaline phosphatase.

30

6.   The method of claim 1, wherein the biological
sample is a sample from the peripheral blood of a
subject.

35   7.   The method of claim 1, wherein the biological
sample is a sample from the lymph nodes of a subject.

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8. A method for detecting prostate specific antigen in a biological sample suspected of containing prostate specific antigen which comprises:

5   (a)   extracting mRNA from the sample;

           (b)   contacting the mRNA from step (a) with reverse transcriptase under conditions allowing for the production of cDNA;

10

           (c)   contacting the cDNA from step (b) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein

15   one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12

20   to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,

25   AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT,

30   or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded

35   duplex comprising the cDNA hybridized to the primers;

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    (d)   contacting the double stranded duplex from step (c) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

    (e)   detecting the cDNA from step (d) thereby detecting prostate specific antigen.

9.   The method of claim 8, wherein in step (c) the first primer is 5'-CACAGACACCCCATCCTATC-3' and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

10.   The method of claim 8, wherein the biological sample is a sample from the peripheral blood of a subject.

11.   The method of claim 8, wherein the biological sample is a sample from the lymph nodes of a subject.

12.   A method of diagnosing prostate cancer in a subject which comprises:

    (a)   obtaining a biological sample from the subject;

    (b)   extracting mRNA from the sample;

    (c)   contacting the mRNA from (b) with reverse transcriptase under conditions allowing for the production of cDNA;

    (d)   contacting the cDNA from step (c) with a pair of reverse transcriptase polymerase chain reaction oligonucleotide primers capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein

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one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(e)   contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f)   detecting the cDNA from step (e) thereby diagnosing prostate cancer in the subject.

13.   The method of claim 12, wherein in step (c) the first primer is 5'-CACAGACACCCCATCCTATC-3' and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

14.   The method of claim 12, wherein at least one of the primers in the set of reverse transcriptase

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polymerase chain reaction primers is covalently linked
to a suitably modified digoxigenin molecule.

15.   The method of claim 14, wherein the detection of
cDNA in step (f) comprises detection of the suitably
modified digoxigenin molecule with a detectably
labeled antibody which specifically binds to the
suitably modified digoxigenin molecule.

16.   The method of claim 15, wherein the detectably
labeled antibody is labeled with an enzyme.

17.   The method of claim 16, wherein the enzyme is
alkaline phosphatase.

18.   The method of claim 12, wherein the biological
sample is a sample from the peripheral blood of a
subject.

19.   The method of claim 12, wherein the biological
sample is a sample from the lymph nodes of a subject.

20.   A method of determining the stage of prostate
cancer in a subject which comprises:

      (a) obtaining a biological sample from the
subject;

      (b) extracting mRNA from the sample;

      (c) contacting the mRNA from (b) with reverse
           transcriptase under conditions allowing for
           the production of cDNA;

      (d) contacting the cDNA from step (c) with a
           pair of reverse transcriptase polymerase
           chain reaction oligonucleotide primers

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capable of specifically hybridizing with DNA encoding prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC; under conditions allowing for hybridization of both primers to any cDNA encoding prostate specific antigen so as to obtain a double stranded duplex comprising the cDNA hybridized to the primers;

(e)　contacting the double stranded duplex from step (d) with DNA polymerase under conditions allowing for the extension of the primers to produce cDNA encoding prostate specific antigen; and

(f)　detecting the cDNA from step (e) thereby determining the stage of prostate cancer in the subject.

21. The method of claim 20, wherein in step (d) the first primer is 5'-CACAGACACCCCATCCTATC-3' and the second primer is 5'-GATGACTCCAGCCACGACCT-3'.

22.   The method of claim 20, wherein at least one of
the primers in the pair of reverse transcriptase
polymerase chain reaction oligonucleotide primers is
covalently linked to a suitably modified digoxigenin
5   molecule.

23.   The method of claim 22, wherein the detection of
cDNA in step (f) comprises detection of the suitably
modified digoxigenin molecule with a detectably
10   labeled antibody which specifically binds to the
suitably modified digoxigenin molecule.

24.   The method of claim 23, wherein the detectably
labeled antibody is labeled with an enzyme.
15

25.   The method of claim 23, wherein the enzyme is
alkaline phosphatase.

26.   The method of claim 20, wherein the biological
20   sample is a sample from the peripheral blood of a
subject.

27.   The method of claim 20, wherein the biological
sample is a sample from the lymph nodes of a subject.
25

28.   An oligonucleotide 12 to 30 nucleotides in length
and having the sequence 5'-X-CACCCCATCCTA-Y-3' wherein
each of X and Y may be present or absent, but if
present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA,
30   AACACAGA,   TAACACAGA,   ATAACACAGA,   AATAACACAGA,
AAATAACACAGA, or CAAATAACACAGA; and Y is T, TC, TCT,
TCTG, TCTGT, or TCTGTG.

29.   The oligonucleotide of claim 28 having the
35   nucleotide sequence 5'-CACAGACACCCCATCCTATC-3'.

30.   The oligonucleotide of claim 28 or 29 covalently

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linked to a suitably modified digoxigenin molecule.

31.   An oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X' and Y' may be present or absent, but if present X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC.

32.   The oligonucleotide of claim 31 having the nucleotide sequence 5'-GATGACTCCAGCCACGACCT-3'.

33.   The oligonucleotide of claim 31 or 32 covalently linked to a suitably modified digoxigenin molecule.

34.   A pair of reverse transcriptase polymerase chain reaction oligonucleotide primers for detecting the expression of the prostate specific antigen, wherein one such primer is an oligonucleotide 12 to 30 nucleotides in length and having the sequence 5'-X-CACCCCATCCTA-Y-3' and the second such primer is an oligonucleotide 12 to 30 nucleotides in length and having the nucleotide sequence 5'-X'-TCCAGCCACGAC-Y'-3' wherein each of X, X', Y, and Y' may be present or absent, but if present X is A, GA, AGA, CAGA, ACAGA, CACAGA, ACACAGA, AACACAGA, TAACACAGA, ATAACACAGA, AATAACACAGA, AAATAACACAGA, or CAAATAACACAGA; X' is C, AC, GAC, TGAC, ATGAC, GATGAC, TGATGAC, GTGATGAC, GGTGATGAC, AGGTGATGAC or CAGGTGATGAC; Y is T, TC, TCT, TCTG, TCTGT, or TCTGTG; and Y' is C, CT, CTC, CTCA, CTCAT, CTCATG, or CTCATGC.

35.   The pair of reverse transcriptase polymerase chain reaction oligonucleotide primers of claim 34, wherein the first such primer is 5'-CACAGACACCCCATCCTATC-3' and the second such primer is
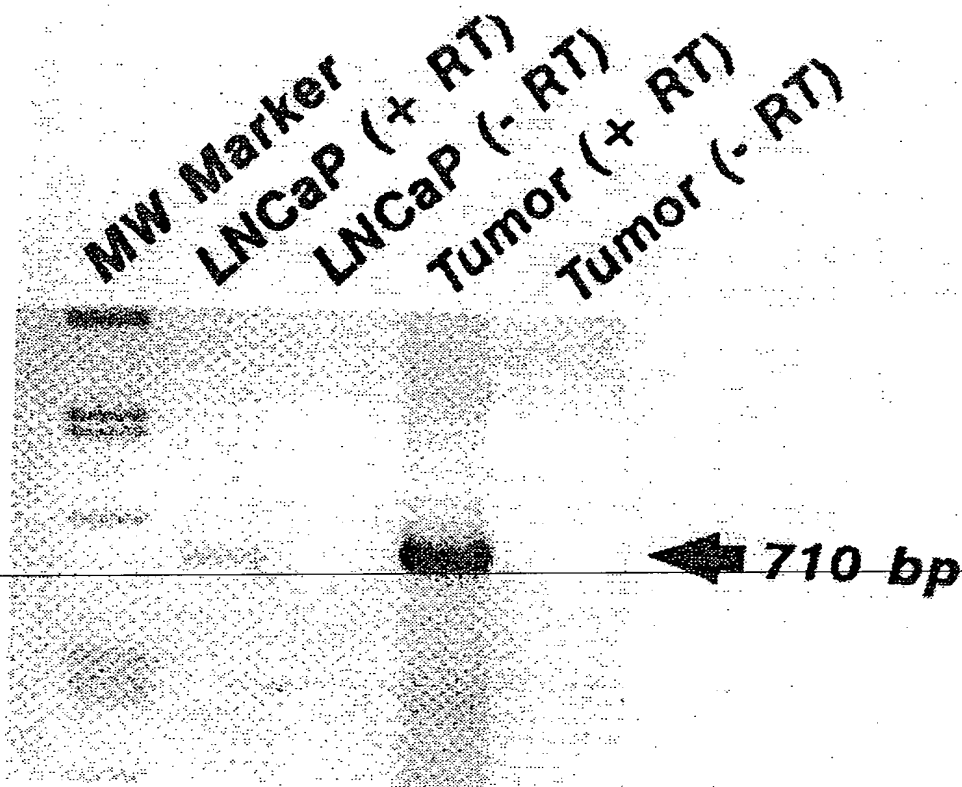
-80-

5'-GATGACTCCAGCCACGACCT-3'.

36. The pair of reverse transcriptase polymerase
chain reaction oligonucleotide primers of claim 34 or
35, wherein at least one of the primers is covalently
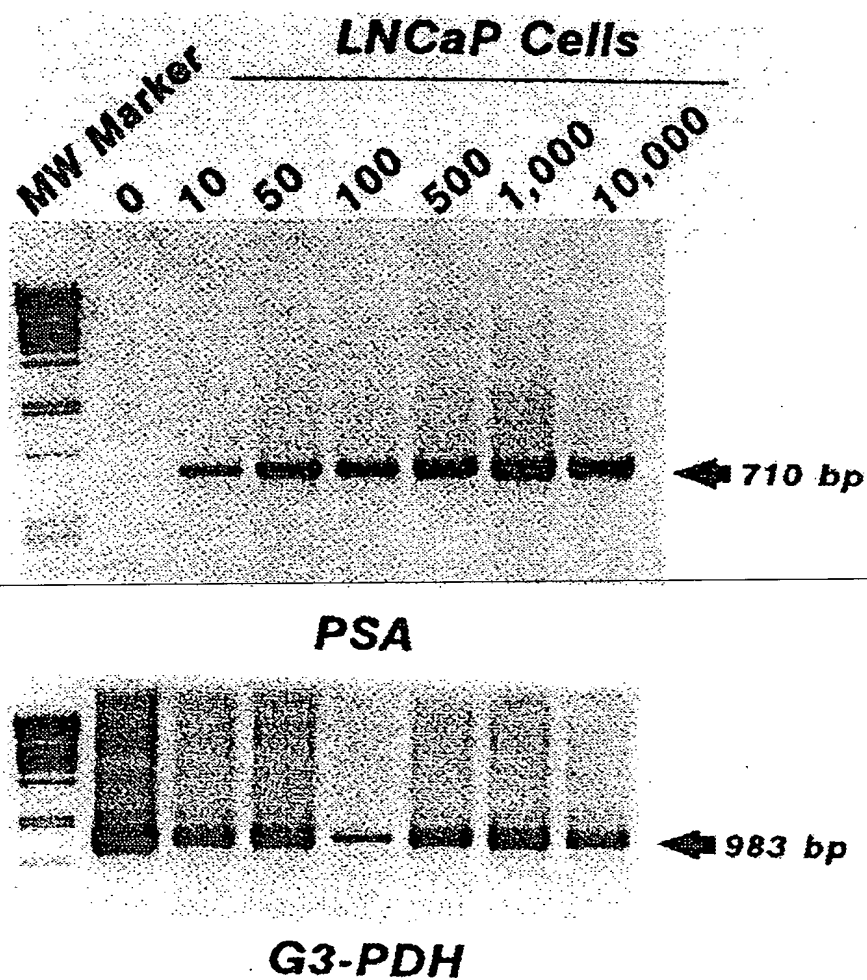linked to a suitably modified digoxigenin molecule.

FIGURE 1

FIGURE 2
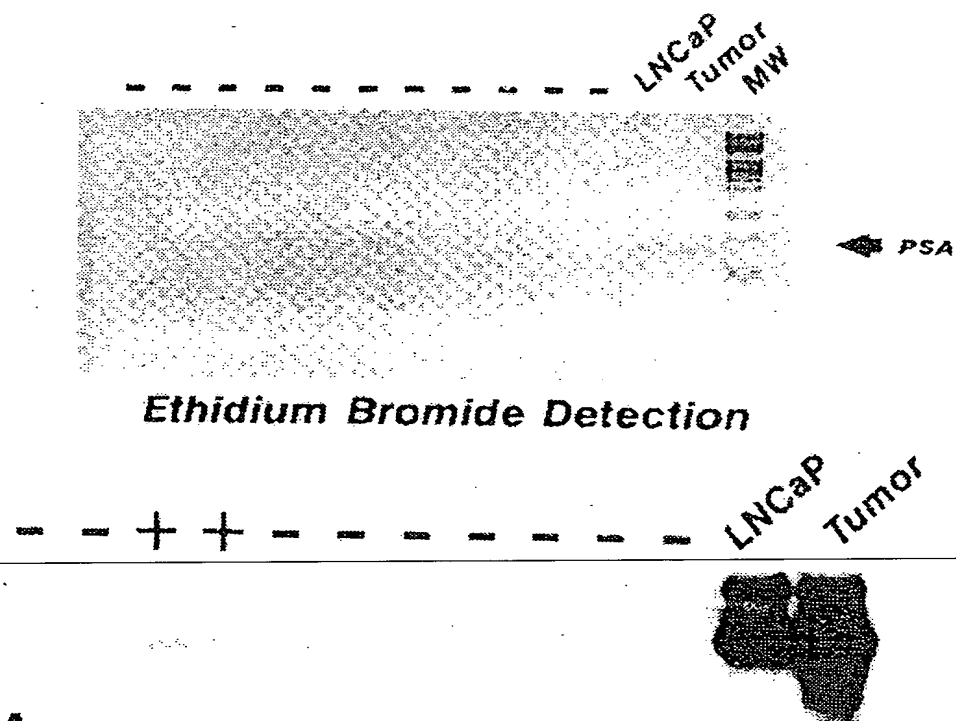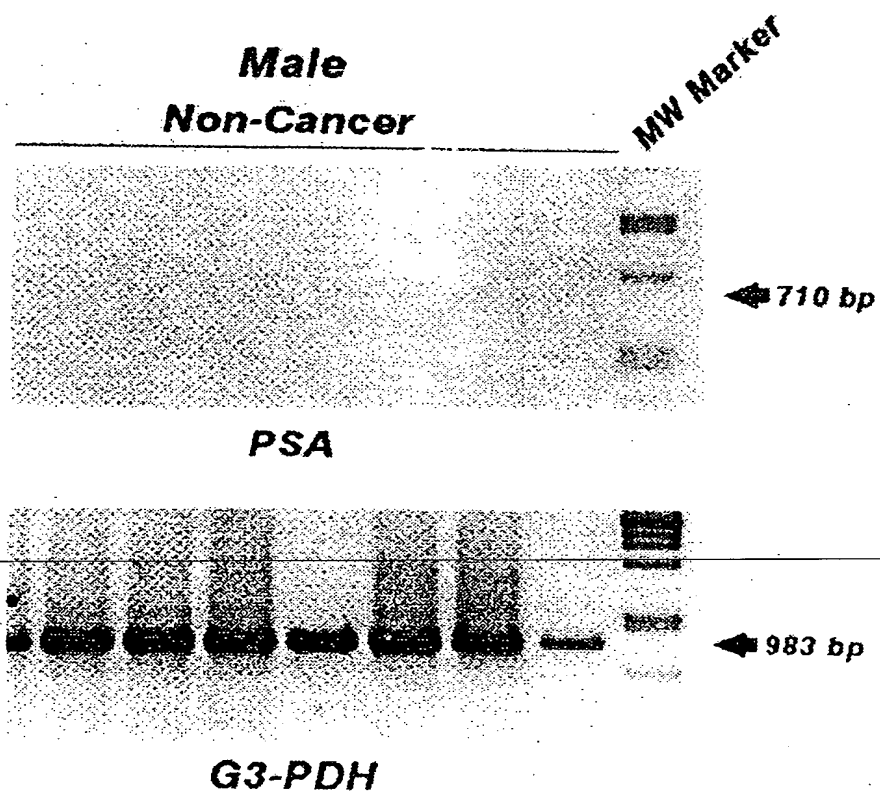

PSA

G3-PDH

FIGURE 3

Ethidium Bromide Detection

A   **Enhanced Detection**

FIGURE 4

Male

Non-Cancer

MW Marker

◄ *710 bp*

PSA

◄ *983 bp*

G3-PDH

FIGURE 5

PSA

G3-PDH
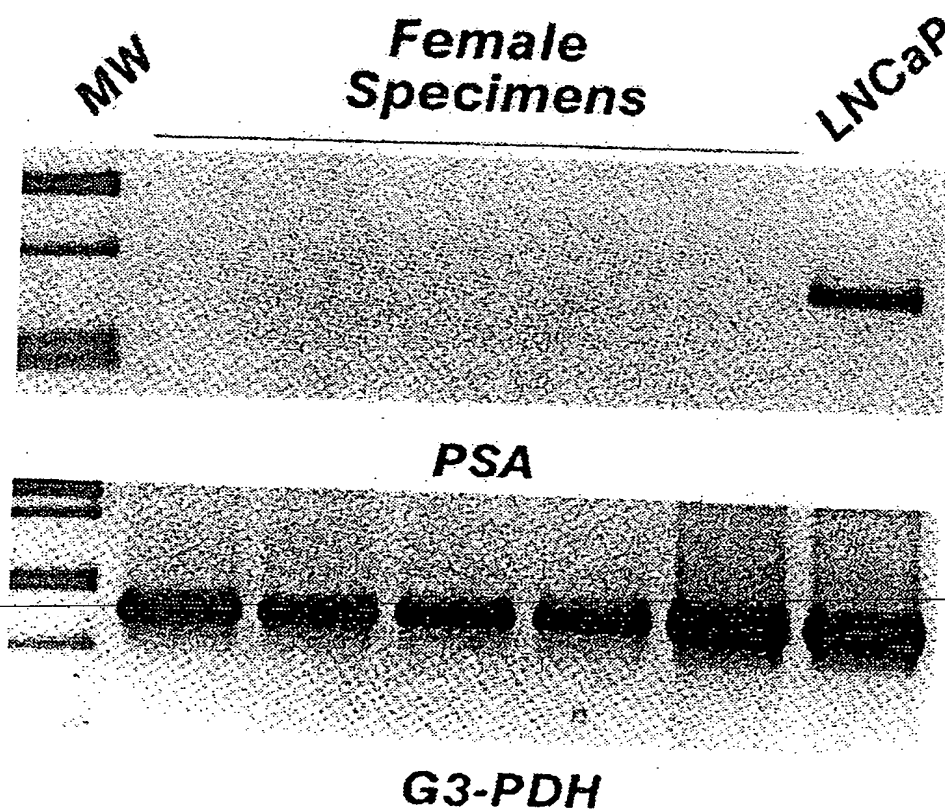
FIGURE 6-1

| FIGURE 6-1 |
| FIGURE 6-2 |
| FIGURE 6-3 |
| FIGURE 6-4 |
| FIGURE 6-5 |

```
AAGTTCCCT   TCTCCCAGTC  CAAGACCCCA  AATCACCACA  AAGGACCCAA  TCCCCAGACT   60
CAAGATATGG  TCTGGGCGCT  GTCTTGTGTC  TCCTACCCTG  ATCCCTGGGT  TCAACTCTGC  120
TCCCAGAGCA  TGAAGCCTCT  CCACCAGCAC  CAGCCACCAA  CCTGCAAACC  TAGGGAAGAT  180
TGACAGAATT  CCCAGCCTTT  CCCAGCTCCC  CCTGCCCATG  TCCCAGGACT  CCCAGCCTTG  240
```

FIGURE 6-2

```
GTTCTCTGCC CCCGTGTCTT TTCAAACCCA CATCCTAAAT CCATCTCCTA TCCGAGTCCC   300

CCAGTTCCTC CTGTCAACCC TGATTCCCCT GATCTAGCAC CCCCTCTGCA GGTGCTGCAC   360

CCCTCATCCT GTCTCCGG ATT GTG GGA GGC TGG GAG TGC GAG AAG CAT TCC    410
                    Ile Val Gly Gly Trp Glu Cys Glu Lys His Ser
                     1           5              10

CAA CCC TGG CAG GTG CTT GTA CCC TCT CGT GGC AGG GCA GTC TGC GGC    458
Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly Arg Ala Val Cys Gly
            15              20              25

GGT GTT CTG GTG CAC CCC CAG TGG GTC CTC ACA GCT ACC CAC TGC ATC    506
Gly Val Leu Val His Pro Gln Trp Val Leu Thr Ala Thr His Cys Ile
        30              35              40

AGG AAC AAA AGC GTG ATC TTG CTG GGT CGG CAC AGC CTG TTT CAT CCT    554
Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His Ser Leu Phe His Pro
        45              50              55

GAA GAC ACA GGC CAG GTA TTT CAG GTC AGC CAC AGC TTC CCA CAC CCG    602
Glu Asp Thr Gly Gln Val Phe Gln Val Ser His Ser Phe Pro His Pro
    60              65              70              75
```

FIGURE 6-3

```
CTC TAC GAT ATG AGC CTC CTG AAG AAT CGA TTC CTC AGG CCA GGT GAT    650
Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe Leu Arg Pro Gly Asp
            80              85              90

GAC TCC AGC CAC GAC CTC ATG CTC CGC CTG TCA GAG CCT GCC GAG        698
Asp Ser Ser His Asp Leu Met Leu Arg Leu Ser Glu Pro Ala Glu
            95              100             105

CTC ACG GAT GCT ATG AAG GTC ATG GAC CTG CCC ACC CAG GAG CCA GCA    746
Leu Thr Asp Ala Met Lys Val Met Asp Leu Pro Thr Gln Glu Pro Ala
            110             115             120

CTG GGG ACC ACC TGC TAC GCC TCA GGC TGG GGC AGC ATT GAA CCA GAG    794
Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly Ser Ile Glu Pro Glu
            125             130             135

GAG TTC TTG ACC CCA AAG AAA CTT CAG TGT GTG GAC CTC CAT GTT ATT    842
Glu Ph  Leu Thr Pro Lys Lys Leu Gln Cys Val Asp Leu His Val Ile
            140             145             150             155
```

FIGURE 6-4

```
TCC AAT GAC GTG TGT GCG CAA GTT CAC CCT CAG AAG GTG ACC AAG TTC    890
Ser Asn Asp Val Cys Ala Gln Val His Pro Gln Lys Val Thr Lys Phe
        160                 165                 170

ATG CTG TGT GCT GGA CGC TGG ACA GGG GGC AAA AGC ACC TGC TCG GGT    938
Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys Ser Thr Cys Ser Gly
            175                 180                 185

GAT TCT GGG GGC CCA CTT GTC TGT AAT GGT GTG CTT CAA GGT ATC ACG    986
Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val Leu Gln Gly Ile Thr
            190                 195                 200

TCA TGG GGC AGT GAA CCA TGT GCC CTG CCC GAA AGG CCT TCC CTG TAC    1034
Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu Arg Pro Ser Leu Tyr
            205                 210                 215

ACC AAG GTG GTG CAT TAC CGG AAG TGG ATC AAG GAC ACC ATC GTG GCC    1082
Thr Lys Val Val His Tyr Arg Lys Trp Ile Lys Asp Thr Ile Val Ala
        220                 225                 230                 235

AAC CCC TGAGCACCCC TATCAACTCC CTATTGTAGT AAACTTGGAA CCTTGGAAAT      1138
Asn Pro

GACCAGGCCA AGACTCAGGC CTCCCCAGTT CTACTGACCT TTGTCCTTAG GTGTGAGGTC   1198
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FIGURE 6-5

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CAGGGTTGCT AGGAAAAGAA ATCAGCAGAC ACAGGTGTAG ACCAGAGTGT TTCTTAAATG   1258

GTGTAATTTT GTCCTCTCTG TGTCCTGGGG AATACTGGCC ATGCCTGGAG ACATATCACT   1318

CAATTCTCT GAGGACACAG ATAGGATGGG GTGTCTGTGT TATTTGTGGG GTACAGAGAT    1378

GAAAGAGGGG TGGGATCCAC ACTGAGAGAG TGGAGAGTGA CATGTGCTGG ACACTGTCCA   1438

TGAAGCACTG AGCAGAAGCT GGAGGCACAA CGCACCAGAC ACTCACAGCA AGGATGGAGC   1498

TGAAAACATA ACCCACTCTG TCCTGGAGGC ACTGGGAAGC CTAGAGAAGG CTGTGAACCA   1558

AGGAGGGAGG GTCTTCCTTT GGCATGGGAT GGGGATGAAG TAAGGAGAGG GACTGACCCC   1618

CTGGAAGCTG ATTCACTATG GGGGGAGGTG TATTGAAGTC CTCCAGACAA CCCTCAGATT   1678

TGATGATTTC CTAGTAGAAC TCACAGAAAT AAAGAGCTGT TATACTGTGA A            1729
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04

US CL :435/6; 91.2; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 91.2; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG: Medline, CA, World Patents, Biosis

search terms: prostate specific antigen; PCR or polymerase chain reaction

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 53, issued 15 November 1993, T. Deguchi et al, "Detection of Micrometastatic Prostate Cancer Cells in Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reaction", pages 5350-5354, especially page 5350.	1-36
Y	Nucleic Acids Research, Volume 17, No. 5, issued March 1989, M. Digby et al, "Human Prostate Specific Antigen (PSA) Gene: Structure and Linkage to the Kallikrein-Like Gene, hGK-1", page 2137, see entire document.	1-36

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 MAY 1995	08 JUN 1995

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer _Deborah Freen_ Carla Myers
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biotechniques, Volume 8, No. 6, issued 1990, J. J. Lanzillo, "Preparation of Digoxigenin-Labeled Probes by the Polymerase Chain Reaction", pages 620-622, see entire document.	1-7, 14-17, 22-25, 30, 33, 36